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Anniina Keskitalo (née Rintala)

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To my family

ABSTRACT

Anniina Keskitalo (née Rintala)
Gut microbes in the prevention and onset of metabolic disorders

University of Turku, Faculty of Medicine, Institute of Biomedicine, Medical Microbiology and Immunology, Turku Doctoral Programme of Molecular Medicine (TuDMM)

Annales Universitatis Turkuensis, Medica - Odontologica, Turku, 2019

Gut microbes are suggested to play an essential role in maintaining human health, and unfavorable alterations in gut microbiota have been associated with several human diseases and disorders. Despite the significant advancements in analysis tools, several sources of uncertainty still exist in the gut microbiota composition analyses, limiting the reproducibility and comparability of the results from distinct gut microbiota studies.

The first main aim of this study was to set up a functional high-throughput pipeline for 16S rRNA gene sequencing -based gut microbiota composition analysis. This was achieved by evaluating the effects of fecal sample processing, i.e. two sample storage conditions and five DNA extraction kits, along with two different 16S rRNA gene sequencing protocols. The second main aim of this study was to explore the role of gut microbiota in the onset of obesity-related metabolic disorders such as non-alcoholic fatty liver disease. This was done by studying the effects of intragastric administration of two distinct gut microbes, *Faecalibacterium prausnitzii* and *Enterobacter cloacae*, on host health and metabolism of high fat diet -fed C57BL/6N mice.

The results of this study showed that the 16S rRNA gene sequencing protocol had a significant effect on the analysis results. Meanwhile, the effect of sample pre-processing was more modest, yet still potentially important. These results indicate that careful design and adequate method optimization are required in order to produce reliable 16S rRNA gene sequencing results. Moreover, the results of this study revealed that the intragastric *F. prausnitzii* administration appeared to protect the C57BL/6N mice from liver steatosis, whereas the intragastric administration of *E. cloacae* seemed to induce liver damage. Further studies are needed in order to clarify the underlying mechanisms and to fully elucidate the possible therapeutic potential of *F. prausnitzii*.

Keywords: gut microbiota, 16S rRNA gene sequencing, metabolic disorders, non-alcoholic fatty liver disease

TIIVISTELMÄ

Anniina Keskitalo (née Rintala)
Suolistomikrobiston rooli lihavuuteen liittyvien aineenvaihduntahäiriöiden synnyssä ja ehkäisyssä

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen mikrobiologia ja immunologia, Turun molekyylilääketieteen tohtoriohjelma (TuDMM)

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Suolistobakteereilla uskotaan olevan merkittävä vaikutus ihmisen terveydelle, ja suolistomikrobiston epätasapaino on yhdistetty moniin eri sairauksiin ja oireyhtymiin. Mikrobistotutkimuksessa käytettävät analyysimenetelmät ovat viimeisen vuosikymmenen aikana kehittyneet merkittävästi, mutta analyysitulokset ovat yhä vahvasti menetelmäriippuvaisia, mikä heikentää tutkimusten toistettavuutta sekä tulosten luotettavuutta ja vertailukelpoisuutta.

Tämän tutkimuksen ensimmäisenä päätavoitteena oli pystyttää toimivat menetelmät 16S rRNA -geenisekvensointiin perustuvalle mikrobistoanalytiikalle. Tutkimuksessa vertailtiin viittä eri DNA-eristysmenetelmää sekä kahta eri sekvensointiprotokollaa, ja selvitettiin ulostenäytteiden pakastuksen vaikutusta mikrobistoanalyysin tuloksiin. Tutkimuksen toisena päätavoitteena oli kartoittaa hiirimallin avulla suolistomikrobiston roolia ei-alkoholiperäisen rasvamaksan synnyssä. Tutkimuksessa selvitettiin, miten suun kautta tapahtuva *Faecalibacterium prausnitzii*- tai *Enterobacter cloacae* -annostelu vaikutti korkearasvaista ravintoa syövien C57BL/6N-hiirten terveyteen ja aineenvaihduntaan.

Tutkimuksen tulokset osoittivat, että 16S rRNA -geenisekvensointimenetelmällä oli merkittävä vaikutus mikrobistoanalyysista saataviin tuloksiin. Näytteiden esikäsittelyn vaikutus oli huomattavasti vähäisempi, mutta kuitenkin tulosten vertailukelpoisuuden kannalta mahdollisesti merkityksellinen. Tulokset osoittavat, että huolellinen suunnittelu ja asianmukainen menetelmäoptimointi ovat välttämättömiä luotettavien 16S rRNA -geenisekvensointituloksen tuottamiseksi. Lisäksi tämän tutkimuksen tulokset osoittivat, että *F. prausnitzii* -annostelu vaikutti positiivisesti hiirten aineenvaihduntaan ja vähensi hiirten maksan rasvoittumista, kun taas *E. cloacae* -annostelu aiheutti maksavaurioita. *F. prausnitzii* -suolistobakteerin mahdollista terveyttä edistävää potentiaalia ja taustalla olevia tekijöitä tulisi selvittää tarkemmissa jatkotutkimuksissa.

Avainsanat: suolistomikrobisto, 16S rRNA geenisekvensointi, metabolinen oireyhtymä, ei-alkoholiperäinen rasvamaksa

TABLE OF CONTENTS

ABBREVIATIONS

Acc	Acetyl coenzyme carboxylase
ALT	Alanine transaminase
AMP	Antimicrobial peptide
AST	Aspartate transaminase
BMI	Body mass index
bp	Base pair
cDNA	Complementary DNA
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FDR	False discovery rate
FFA	Free fatty acid
FMT	Fecal microbiota transplantation
G+C	Guanine + Cytosine
GF	Germ-free
GI	Gastrointestinal
GXT	GXT Stool Extraction Kit VER 2.0 (Hain Lifescience GmbH)
HFD	High-fat diet
HSL	Hormone sensitive lipase
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IL	Interleukin
IR	Insulin resistance
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MOB	PowerFecal DNA Isolation Kit (MO BIO Laboratories, Inc.)
MP	MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche Diagnostics GmbH)
mRNA	Messenger RNA
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction

PGC1α	Peroxisome proliferator -activated receptor coactivator 1α
QIA	QIAamp DNA Stool Mini Kit (QIAGEN GmbH)
QIAF	QIAamp Fast DNA Stool Mini Kit (QIAGEN GmbH)
qPCR	Real-time quantitative PCR
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
SAT	Subcutaneous adipose tissue
SCFA	Short-chain fatty acid
SPF	Specific-pathogen-free
T2D	Type 2 diabetes
TAG	Triacylglycerol (triglyceride)
TLR	Toll-like receptor
tRNA	Transfer RNA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VAT	Visceral adipose tissue
YCFAG agar	Yeast extract, casitone, fatty acid, and glucose agar

LIST OF ORIGINAL PUBLICATIONS

This study was based on the following publications, which are referred to in the text by Roman numerals (I - III). In addition, unpublished data are included.

I Rintala A, Pietilä S, Munukka E, Eerola E, Pursiheimo J-P, Laiho A, Pekkala S, Huovinen P. Gut microbiota analysis results are highly dependent on the 16S rRNA gene target region, whereas the impact of DNA extraction is minor. *Journal of Biomolecular Techniques*. 2017; 8:19-30. doi: 10.7171/jbt.17-2801-003

II Munukka E*, Rintala A*, Toivonen R, Nylund M, Yang B, Takanen A, Hänninen A, Vuopio J, Huovinen P, Jalkanen S, Pekkala S. *Faecalibacterium prausnitzii* treatment improves hepatic health and reduces adipose tissue inflammation in high-fat fed mice. *The ISME Journal*. 2017; 11:1667-1679; doi: 10.1038/ismej.2017.24. *Equal contribution

III Keskitalo A, Munukka E, Toivonen R, Hollmén M, Kainulainen H, Huovinen P, Jalkanen S, Pekkala S. *Enterobacter cloacae* administration induces hepatic damage and subcutaneous fat accumulation in high-fat diet fed mice. *PLoS ONE.* 2018; 13(5): e0198262. doi: 10.1371/journal.pone.0198262

1 INTRODUCTION

Human gastrointestinal (GI) tract is densely colonized by microbial inhabitants, and the balance and welfare of these microbes appears to be crucial for human health (Sekirov et al., 2010, Wu et al., 2015, Lynch & Pedersen, 2016). During the past decade, significant methodological and technological advancements have provided completely new approaches to study the composition and functions of the gut bacteria (Klindworth et al., 2013, Lepage et al., 2013, Mandal et al., 2015), and consequently, the amount of information concerning the intestinal microbiota composition, along with possible host-bacterial interactions, has increased enormously (Chow et al., 2010, Tremaroli & Bäckhed, 2012, McLean et al., 2015, Stenman et al., 2015, Gérard, 2016, Zhernakova et al., 2016, Jackson et al., 2018).

Obesity has become a global health burden; the worldwide number of obese adult individuals is already reaching 2 billion, and the prevalence is constantly increasing (World Health Organization, 2017). As a consequence, the incidence of obesity-associated metabolic disorders has also increased. For example, non-alcoholic fatty liver disease (NAFLD), the so-called hepatic manifestation of metabolic syndrome, has become the most common liver disorder in the Western world (Henao-Mejia et al., 2013). Even though obesity and the associated metabolic disorders are generally considered to result from unhealthy diet and lifestyle, constantly accumulating evidence indicates that gut microbiota may play an important role in the development of the obesity-related metabolic disorders such as insulin resistance (IR), Type 2 diabetes (T2D), and NAFLD (Shen et al., 2013, Cani & Delzenne, 2014, Alard et al., 2016, Xie et al., 2016).

Faecalibacterium prausnitzii is a commensal gut bacterium that conceivably supports host health and serves as a potential indicator of a healthy human gut (reviewed in Ferreira-Halder et al., 2017). By contrast, family *Enterobacteriaceae,* and particularly *Enterobacter cloacae* strain B29, have been associated with obesity and hepatic damage (Fei & Zhao, 2013, Munukka et al., 2016). The underlying mechanisms behind the reported associations remain largely unknown. This study aimed at illustrating the mechanisms underlying the proposed health-promoting or -compromising effects of these gut bacteria by examining the effects of intragastric administration of either *F. prausnitzii* (ATCC® 27766™) or *E. cloacae* subsp. *cloacae* (ATCC® 13047™) on host health and metabolism of high-fat diet (HFD) fed C57BL/6N mice.

2 REVIEW OF LITERATURE

2.1 Gut Microbiota

Human gut harbors one of the most complex microbial ecosystems on Earth, comprised of trillions of microbes (Whitman et al., 1998, Sekirov et al., 2010, Huttenhower et al., 2012, Sankar et al., 2015). Term 'gut microbiota' refers to all the GI tract microbes, including bacteria, archaea, eukaryotes, and viruses (Zoetendal et al., 2008, Rajilić-Stojanović & de Vos, 2014). Until recently, bacteria were thought to represent the most diverse and abundant microbial group residing in the human GI tract (Rajilić-Stojanović & de Vos, 2014). Recent studies have, however, reported that intestinal viruses, specifically bacteriophages, represent a remarkably diverse population that may actually clearly outnumber the bacterial inhabitants of the gut (Bakhshinejad & Ghiasvand, 2017, Cani, 2018). The overall diversity and clinical significance of the GI tract bacteriophages still remain largely unknown, but it has been stated that the phages may play an important role in human health and disease (Bakhshinejad & Ghiasvand, 2017, Cani, 2018). This literature review focuses on the bacterial population of the gut microbiota, which at present is the most comprehensively studied microbial group colonizing the human gut (Cani, 2018).

The bacterial community inhabiting the human GI tract is dominated by anaerobic bacteria (Rajilić-Stojanović & de Vos, 2014). The proportion of bacterial cells increases dramatically from stomach to colon, and especially colon maintains an exceptionally diverse bacterial population (Sekirov et al., 2010, Sankar et al., 2015). In fact, colon microbiota has been suggested to be one of the most important microbial communities within the host, and the composition and functions of the ecosystem have thus been particularly intensively studied (Rajilić-Stojanović & de Vos, 2014, Sankar et al., 2015, Cani, 2018). The most predominant bacterial phyla residing in the human GI tract are *Bacteroidetes* and *Firmicutes*; these two phyla generally constitute more than 90 % of all gut bacteria in healthy adult individuals (Eckburg et al., 2005, Arumugam et al., 2011). *Bacteroidetes* is comprised of Gram-negative bacteria, whereas *Firmicutes* is comprised mainly of Gram-positive bacteria. Other prevalent phyla include *Actinobacteria* and *Proteobacteria*, followed by sparser groups such as *Fusobacteria* and *Verrucomicrobia* (Table 1; Zoetendal et al., 2008, Arumugam et al., 2011, Rajilić-Stojanović & de Vos, 2014).

Table 1. The main bacterial groups present in the human GI tract microbiota (for a detailed review, see Rajilić-Stojanović & de Vos, 2014)

Phylum	General properties	Examples of genera and species
Firmicutes	• Extremely diverse phylum including both commensal bacteria and opportunistic pathogens; class *Clostridia* includes species dominant in the lower GI tract, whereas class *Bacilli* includes species dominant in the upper parts of the GI tract. • Mainly Gram-positive bacilli/cocci • Several spore-forming species • Significant role in food metabolism, several important butyrate producers • Phylum includes several commercially applied probiotics and potential future probiotics, but also important pathogens such as *C. difficile*	*Clostridia:* *Clostridium: C. perfringens* *Eubacterium: E. rectale* *Faecalibacterium: F. prausnitzii* *Ruminococcus* spp., *Blautia* spp., *Dorea* spp. *Bacilli:* *Bacillus: Bacillus cereus* *Lactobacillus: L. acidophilus, L. casei* *Streptococcus* spp., *Enterococcus* spp., *Staphylococcus* spp., *Veillonella* spp.
Bacteroidetes	• Substantially diverse phylum including both commensal bacteria and opportunistic pathogens • Gram-negative bacilli • Some species possess ability to degrade complex polysaccharides • Broad metabolic potential, health effects not fully elucidated	*Bacteroides: B. fragilis, B. dorei, B. thetaiotaomicron, B. vulgatus* *Prevotella: P. melaninogenica*
Actinobacteria	• Gram-positive bacteria, typically difficult to lyse • High G+C content in DNA • Often underrepresented in 16S rRNA gene sequencing -based gut microbiota surveys • Several important carbohydrate fermenters • Certain *Bifidobacterium* strains are considered as health-beneficial and are commercially applied as probiotics	*Bifidobacterium: B. adolescentis, B. breve, B. longum* *Collinsella: C. aerofaciens*
Proteobacteria	• Gram-negative bacilli, divided into several classes based on 16S rRNA sequence • Relatively low overall abundance in a healthy gut • Accurate genus level identification not feasible with current 16S rRNA gene sequencing -based gut microbiota composition analysis tools • *Enterobacteriaceae*, belonging to class *γ-Proteobacteria* , possess both flagella and LPS, and are thus considered as pro-inflammatory • Phylum includes a wide variety of important GI tract pathogens, but certain species have also been commercially applied as probiotics	*β-Proteobacteria:* *Suttetella* spp. *γ-Proteobacteria*: *Enterobacter: E. cloacae* *Escherichia: E. coli* *Haemophilus* spp., *Pseudomonas* spp. *δ-Proteobacteria:* *Desulfovibrio* spp. *ε-Proteobacteria:* *Campylobacter* spp., *Helicobacter* spp.
Fusobacteria	• Gram-negative, non-spore-forming bacilli • Generally considered as pro-inflammatory	*Fusobacterium: F. nucleatum*
Verrucomicrobia	• Gram-negative, non-spore-forming • *A. muciniphila* is a mucin degrader that is suggested to be important for host health	*Akkermansia: A. muciniphila*

Altogether, representatives from more than 10 different bacterial phyla have been detected from human GI tract samples, and it should be noted that most of these phyla are comprised of extremely large collections of bacterial species that possess divergent properties and functions (Rajilić-Stojanović & de Vos, 2014, Hugon et al., 2015, Marchesi et al., 2016). Furthermore, comprehensive coverage of the human gut microbiota composition and diversity is yet to be achieved; even though more than a thousand distinct human GI tract micro-organisms have already been isolated and identified, it has been predicted that part of the bacterial inhabitants may still remain completely uncharacterized (Lagier et al., 2012, Rajilić-Stojanović & de Vos, 2014, Sankar et al., 2015, Browne et al., 2016).

The composition of gut microbiota is host specific and evolving throughout an individual's life (Lozupone et al., 2012, Kundu et al., 2017). Until recently, human placenta and developing fetus have been regarded as sterile, while the initial microbe contact of the offspring has been assumed to occur during birth. Several recent studies have, however, challenged this sterility dogma by reporting findings of microbes, or at least traces of microbial DNA, in womb, placenta, umbilical cord blood, and amniotic fluid (Rautava et al., 2012, Zheng et al., 2015, Verstraelen et al., 2016). These findings suggest that, instead of maintaining a completely sterile environment for the developing offspring, the human placenta may act as a selective barrier allowing certain maternal microbes to colonize the fetus *in utero* (Funkhouser & Bordenstein, 2013, Kundu et al., 2017). Nevertheless, as specimens with zero/low bacterial biomass are highly predisposed to external contamination during sample collection and analysis, it has been suggested that the results of these studies could be biased (Kundu et al., 2017). Thus, no firm consensus on the issue has been reached to date. In either case, the major colonization of infant gut microbiota begins at birth (Milani et al., 2017).

Delivery mode and lactation play a significant role in early gut microbiota development (Grölund et al., 1999, Penders et al., 2006, Huurre et al., 2008, Dominguez-Bello et al., 2010), while introduction of solid foods is suggested to be the initial step for more rapid microbiota diversification and maturation (Milani et al., 2017, Kundu et al., 2017). Gut microbiota profile of a three-year-old child already partially resembles the gut microbiota of an adult (Yatsunenko et al., 2012, Cheng et al., 2016), but the microbiota composition and diversity seem to undergo constant gradual development until early adulthood (Hollister et al., 2015, Odamaki et al., 2016). Accordingly, the maturation of human gut microbiota is suggested to proceed in parallel with the development of host intestine (Kundu et al., 2017); the bacterial diversity increases as the intestine elongates with age, finally reaching a total mucosal area of ~32 m^2 on average

(Helander & Fändriks, 2014, Kundu et al., 2017). In adults, the GI tract microbiota is assumed to be a rather steady, yet dynamic, ecosystem (Costello et al., 2009, Chow et al., 2010, Lozupone et al., 2012). Based on recent large population-based studies, the most important underlying factors affecting the adulthood gut microbiota composition include environmental factors such as diet, lifestyle, and medication, along with host-related factors including age, gender, and body mass index (BMI) (Falony et al., 2016, Zhernakova et al., 2016, Haro et al., 2016). Role of genetic or ancestral background, on the other hand, is suggested to be minor (Jackson et al., 2018, Rothschild et al., 2018), although contradictory reports also exist (Goodrich et al., 2014, Lim et al., 2017).

Most gut bacteria are harmless to the host, and humans along with several animals have evolved a symbiotic relationship with the gut microbes (Tremaroli & Bäckhed, 2012, Lepage et al., 2013). Gut bacteria, for example, participate in food digestion (chapter 2.1.1) and have an important role in protecting the host from invading pathogens (chapter 2.1.2). In fact, as the ecosystem constituted by the intestinal bacteria is both metabolically highly active and in constant interaction with the host, it has been stated that the gut microbiota should be referred as an "external organ" that is integrated in the host's metabolism (Bäckhed et al., 2005, Lepage et al., 2013).

2.1.1 Role of gut bacteria in food digestion and metabolism

Gut microbes have an essential role in the food digestion of the host (Tremaroli & Bäckhed, 2012). Gut bacteria, for example, can biosynthesize vitamins from several precursors derived from ingested food, regulate bile acid metabolism, and utilize nutrients that would otherwise remain non-digestible for the host (Bäckhed et al., 2004, Tremaroli & Bäckhed, 2012, Yatsunenko et al., 2012). Accordingly, the intestinal bacteria are able to produce metabolites that are otherwise inaccessible for the host. Dietary polysaccharides, including cellulose, hemicellulose, resistant starches, and plant-derived pectin, are examples of these otherwise inaccessible nutrients; gut microbes can ferment dietary polysaccharides into short-chain fatty acids (SCFAs), mainly acetate, butyrate, and propionate, and at the same time elevate the absorption of monosaccharides (Bäckhed et al., 2004, Sonnenburg & Sonnenburg, 2014). In addition, gut microbes are able to ferment dietary proteins, transform complex plant phytochemicals into simpler metabolites, and convert primary bile acids into secondary bile acids (Louis et al., 2014, Sonnenburg & Bäckhed, 2016). The end products of microbial metabolism can serve as energy supplies for the host metabolism as well as signaling molecules (Tremaroli & Bäckhed, 2012,

Sonnenburg & Bäckhed, 2016). SCFAs, for instance, are important regulatory molecules that can act as histone deacetylase inhibitors or signal through G-protein-coupled receptors (Louis et al., 2014, Sonnenburg & Bäckhed, 2016). In addition, SCFAs are important energy sources for the host and they serve as substrates for host metabolic pathways such as gluconeogenesis (Sonnenburg & Bäckhed, 2016). Especially butyrate works as an important energy source for the colonocytes, and it has been associated with alleviated intestinal inflammation and improved gut epithelial integrity (Hamer et al., 2009). Elevated SCFA production, however, may also lead to excess energy absorption and thus predispose to obesity, and therefore it has been suggested that SCFA-producing gut microbes may have an important role in the host energy metabolism (Bäckhed et al., 2004). Several studies have reported differences in the gut microbiota profiles between obese and lean individuals, supporting the idea that gut microbes may play a central role in the development of obesity and obesity-related metabolic disorders (Cani & Delzenne, 2014, Stenman et al., 2015, Gérard, 2016). On the other hand, diet changes are able to rapidly modify the composition and metabolic functions of the gut microbiota (O'Keefe et al., 2015, Graf et al., 2015). Altogether, the metabolic capacity of the gut microbes is still relatively poorly understood, and gut microbes are likely to produce a vast number of metabolites whose functions or effects on human health still remain to be established (reviewed in Sonnenburg & Bäckhed, 2016). Comprehensive investigation is therefore required in order to determine the overall significance of the gut microbiota -derived metabolites and to more profoundly understand the functional host-microbe interactions (Tremaroli & Bäckhed, 2012, Wu et al., 2015).

2.1.2 Gut barrier function and intestinal dysbiosis

Intestinal epithelium is in a close and constant contact with the gut microbiota. The gut epithelium possesses various mechanisms to restrain bacterial growth, to prevent bacterial adhesion, and to block the dissemination of the microbes and microbial toxins into underlying tissues (Ohland & Macnaughton, 2010, Muniz et al., 2012). These defense mechanisms include, for example, a stratified mucous layer that covers the intestinal epithelial cells, pattern-recognition receptors of the epithelial cells, antimicrobial peptides (AMPs) secreted by Paneth cells, secretory immunoglobulin A (IgA), and epithelial junctional complexes that regulate the permeability between the epithelial cells (Figure 1a). The purpose of this intestinal barrier is, in addition to protecting the host from the invasion of bacterial pathogens, to restrain uncontrolled inflammatory responses

and to maintain a balanced immunological state, i.e. homeostasis, in the gut (McGuckin et al., 2009, Muniz et al., 2012).

Alongside the host factors, commensal gut bacteria play a key role in maintaining the gut homeostasis and preventing the colonization of pathogenic microbes (Chow et al., 2010, Belkaid & Hand, 2014). Commensal gut microbes are constantly interacting with the host innate immune system by providing immunological signals to the intestinal epithelial cells (Cerf-Bensussan & Gaboriau-Routhiau, 2010, Hiippala et al., 2018). The epithelial cells express receptors for microbial-associated molecular patterns, and these receptors, including Toll-like receptors (TLRs) and NOD-like receptors, activate signaling cascades that regulate the proliferation of the epithelial cells as well as the production of AMPs (Cerf-Bensussan & Gaboriau-Routhiau, 2010, Muniz et al., 2012). In addition, the signals from the commensal bacteria are able to activate cells of the adaptive immune system and, for example, induce the secretion of the protective IgA antibodies. AMPs as well as IgA modulate the gut microbiota and prevent the luminal microbes from crossing the epithelial barrier (Chow et al., 2010, Cerf-Bensussan & Gaboriau-Routhiau, 2010, Muniz et al., 2012). This constant interaction between the commensal gut microbes and the host immune system maintains a so-called physiological inflammation in the gut and has a crucial role in maintaining the intestinal homeostasis (Figure 1b).

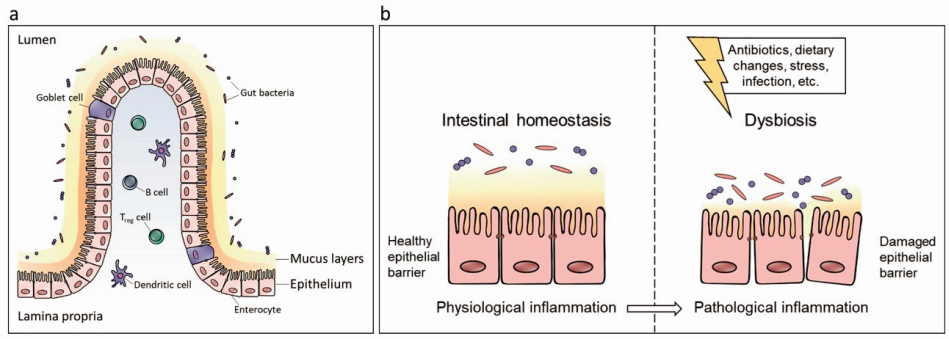

Figure 1. Intestinal barrier function. In a healthy gut, a physical epithelial barrier, together with commensal gut microbiota and immunological feedback mechanisms, works efficiently in avoiding microbial invasion and excessive activation of the host immune responses (a). Certain external triggers disrupting the gut microbiota and the immune balance can, however, lead to increased bacterial adherence, epithelial damage, and elevated entry of bacteria or bacteria-derived endotoxins, along with other molecules, into the intestinal lamina propria (b). This physiological stage called dysbiosis affects the host immune system and promotes the onset of intestinal inflammation. Based on (Cerf-Bensussan & Gaboriau-Routhiau, 2010, Muniz et al., 2012, Belkaid & Hand, 2014).

Even though an individual's gut microbiota is believed to form a relatively stable community, gut microbes undergo continuous selective pressure and are susceptible to both exogenous and endogenous modification (Lozupone et al., 2012, Lynch & Pedersen, 2016). Several external triggers, including dietary changes, medication, and changes in the physiological state of the host, can significantly alter the gut microbiota composition (Lynch & Pedersen, 2016, Falony et al., 2016, Zhernakova et al., 2016). Unfavorable alterations in the gut microbiota can cause disruption of the colonization resistance provided by the commensal bacteria, leading to a physiological state called dysbiosis (Figure 1b) that potentially results in a pathological inflammation and disease (Modi et al., 2014, Graf et al., 2015). In a dysbiotic gut microbiota, the overall bacterial diversity is usually reduced, whereas the proportion of opportunistic pathogens such as endotoxin-producing *γ-Proteobacteria* is increased (Walker & Lawley, 2013). Meanwhile, the abundance of phylum *Firmicutes*, including several important SCFA-producers, is typically reduced (Walker & Lawley, 2013). SCFAs are important energy sources for the colonocytes and the reduced SCFA production may thus disrupt epithelial integrity. Consequently, gut microbiota dysbiosis predisposes the host to metabolic endotoxemia (discussed in Chapter 2.3.3) and systemic inflammation. Accordingly, GI tract microbiota dysbiosis has been associated with several diseases and disorders, including obesity (Turnbaugh et al., 2008, Cani & Delzenne, 2014, Stenman et al., 2015, Gérard, 2016), diabetes (Davis-Richardson et al., 2014, Endesfelder et al., 2014, Miele et al., 2015), rheumatoid arthritis (Eerola et al., 1994, Vaahtovuo et al., 2008, Sandhya et al., 2016), and inflammatory bowel disease (IBD) (Sokol et al., 2006, Muniz et al., 2012, Becker et al., 2015). The use of antimicrobial drugs is a common example of an external trigger that is capable of significantly decreasing the gut microbiota diversity and this way predispose the host to intestinal infections (reviewed in Modi et al., 2014). Simultaneously with constant rise in global antibiotic consumption (Friedrich, 2018), an increasing number of studies are illustrating the impact of antibiotic-induced gut microbiota alterations on human health (reviewed in Francino, 2016). Altogether, a homeostatic state in the human gut seems to require constant interaction and delicate balance between the commensal gut bacteria and the host, and disturbances of the gut microbiota can even lead to comprehensive immune dysfunction or autoimmunity (reviewed in Fung et al., 2012 and Honda & Littman, 2016).

2.1.3 *Faecalibacterium prausnitzii*

One of the most interesting members of gut microbiota is *Faecalibacterium prausnitzii* (*F. prausnitzii*), which is the only identified representative of genus

Faecalibacterium. *F. prausnitzii* is one of the most abundant bacteria in the gut of several vertebrates, including human (Suau et al., 2001, Miquel et al., 2014), swine (Haenen et al., 2013, Foditsch et al., 2014), bovine (Oikonomou et al., 2013, Foditsch et al., 2014), and chicken (Lund et al., 2010). *F. prausnitzii* belongs to *Clostridium leptum* (*C. leptum*) cluster, which further resides under phylum *Firmicutes*. Morphologically, *F. prausnitzii* is a non-motile and non-spore-forming rod of size 0.5 - 0.8 × 2.0 - 14.0 µm (Duncan et al., 2002). The genome length of the bacterium is approximately 3.1 Mbp, and the G+C content of the DNA is around 56 % (Benevides et al., 2017). *F. prausnitzii*, as well as all other members of *C. leptum* cluster, is Gram-positive and strictly anaerobic (Lopez-Siles et al., 2012). The bacterium can, however, appear Gram-negative in staining, and as a matter of fact, *F. prausnitzii* was previously classified into Gram-negative *Fusobacterium* genus (Duncan et al., 2002). The classification and nomenclature were updated, since phylogenetic analyses revealed that the bacterium was obviously more related to *Clostridia* rather than *Fusobacteria* (Wang et al., 1996, Duncan et al., 2002). Based on 16S ribosomal RNA (rRNA) sequence analyses, *F. prausnitzii* species can be clustered into two or three phylogroups (Lopez-Siles et al., 2012, Martin et al., 2017). On the other hand, a recent pangenome analysis suggests that the phylogenetics of the species might be even more complex (Benevides et al., 2017).

Functionally, *F. prausnitzii* is a highly active gut bacterium that appears to have beneficial impact on host health and physiology (Li et al., 2008, Miquel et al., 2014, Miquel et al., 2015, Lopez-Siles et al., 2017). The bacterium, for example, possesses anti-inflammatory properties by inhibiting the production of pro-inflammatory cytokines interleukin (IL)-12 and interferon γ, and by increasing the production of anti-inflammatory IL-10 (Sokol et al., 2008, Qiu et al., 2013). Recently, a Microbial Anti-Inflammatory Protein (MAM), secreted by *F. prausnitzii*, has been identified (Quévrain et al., 2016), and this bioactive molecule is suggested to reduce inflammation by blocking nuclear factor (NF)-κB pathway and by regulating the cytokine production (Quévrain et al., 2016, Breyner et al., 2017). Further, metabolites like salicylic acid may be involved in the anti-inflammatory effects of the bacterium (Miquel et al., 2015). In addition to the regulation of cytokine production, *F. prausnitzii* is suggested to enhance intestinal barrier function by affecting the paracellular permeability of the gut epithelium (Carlsson et al., 2013, Martin et al., 2015). One probable link between the bacterium and the enhanced gut barrier function is the SCFA butyrate; *F. prausnitzii* is one of the main butyrate producers in the gut, and in humans, reduced intestinal *F. prausnitzii* abundance has been associated with low fecal butyrate concentration (Duncan et al., 2004, Benus et al., 2010). Butyrate is the preferred energy source for gut epithelial cells, and it is known to maintain favorable intestinal barrier function and to decrease oxidative stress (Hamer et

al., 2008, Hamer et al., 2009). In addition, butyrate regulates cell proliferation and differentiation, and is suggested to protect against colorectal cancer (Bradburn et al., 1993, Archer et al., 1998, Siavoshian et al., 2000). Along with butyrate, *F. prausnitzii* hydrolyzes starch, inulin, and various fructo-oligosaccharides, which are all known to possess health-beneficial properties for the gut (Duncan et al., 2002).

Reduction in *F. prausnitzii* abundance has been associated with several human diseases and disorders, including obesity (Hippe et al., 2016), metabolic disorders (Munukka et al., 2012), IBD (Sokol et al., 2009, Lopez-Siles et al., 2016), and colorectal cancer (Lopez-Siles et al., 2016). In addition, low *F. prausnitzii* abundance has been linked to high liver fat content (Munukka et al., 2014) and non-alcoholic steatohepatitis (NASH) (Wong et al., 2013). Furthermore, *F. prausnitzii* and its culture supernatant have been shown to ameliorate intestinal inflammation in murine models (Martin et al., 2014, Martin et al., 2015, Huang et al., 2016, Miquel et al., 2016). Due to the numerous beneficial properties of *F. prausnitzii*, it has been proposed that the bacterium could serve as biomarker to assist in the diagnostics of gut disorders and/or act as a next-generation probiotic supplement in the treatment of patients with the above-mentioned disorders and diseases (Lopez-Siles et al., 2017, Martin et al., 2017). However, the metabolic activities of *F. prausnitzii* have been shown to vary between the different strains of the bacterium (Martin et al., 2017), suggesting that the anti-inflammatory capacities may not be equal between different strains. This within-species variation may set challenges on drawing conclusions from the association studies, as the species phylogroups are usually not determined in gut microbiota composition studies.

2.1.4 *Enterobacter cloacae*

Enterobacter cloacae (*E. cloacae*) is a facultative anaerobic gut bacterium belonging to family *Enterobacteriaceae* and genus *Enterobacter*. Morphologically, enterobacteria are Gram-negative, non-spore-forming rods of size 0.6 - 1.0 × 1.2 - 3.0 µm (Gaston, 1988). *E. cloacae* is a motile bacterium with peritrichous flagella (Hormaeche & Edwards, 1960, Keller et al., 1998), and as all members of the genus *Enterobacter*, it is an endotoxin-producing bacterium (Sanders & Sanders, 1997). The genome length of the type strain of the bacterium, *E. cloacae* subspecies *cloacae* ATCC® 13047™, is approximately 5.3 Mbp, and the G+C content of the DNA is 54.7 % (Ren et al., 2010). *E. cloacae* belongs to normal human gut microbiota, but as an opportunistic pathogen, it can also cause a wide range of infections, including urinary tract

infections, bacteremia, and intra-abdominal infections (Sanders & Sanders, 1997, Lee et al., 2002). *E. cloacae* is naturally resistant to amoxicillin and cephalosporins, as it produces chromosomally encoded beta-lactamases (Bush et al., 1995). Interestingly, the abundance of *Enterobacteriaceae* family has been shown to increase in the gut microbiota of patients with NASH or liver cirrhosis (Lu et al., 2011, Chen et al., 2011, Özkul et al., 2017).

Intestinal abundance of *E. cloacae* has been linked to obesity (Fei & Zhao, 2013, Yan et al., 2016). In a case study by Zhao and co-workers, a significant weight loss of a morbidly obese individual was associated with substantial decrease in the intestinal *E. cloacae* B29 load and significant reduction in serum endotoxin levels (Fei & Zhao, 2013). Furthermore, the same *E. cloacae* strain, isolated from the patient, was shown to initiate obesity and to induce significant changes in colon gene expression profiles when introduced to HFD fed germ-free (GF) mice, i.e. mice that lack normal microbiota (Fei & Zhao, 2013, Yan et al., 2016). However, neither HFD nor *E. cloacae* B29 alone seems to induce obesity or cause significant gene-expression alterations in the GF mice (Bäckhed et al., 2007, Yan et al., 2016). Even though the GF mice are not the most optimal model to study the functions of the gut microbes (discussed in chapter 2.4.1), these results indicate that certain endotoxin-producing microbes and HFD may possess a synergistic effect that, possibly through an endotoxin-induced mechanism, contributes to the development of obesity (Fei & Zhao, 2013, Yan et al., 2016).

2.2 Methods to study gut microbiota

In the past decades, associations between gut microbiota and human diseases have been of great interest. In fact, gut microbiota is suggested to serve as an important potential target for future clinical diagnostics and personalized medical treatments (Guinane & Cotter, 2013, Lynch & Pedersen, 2016). The methods to study the gut microbiota composition and functions have advanced significantly during the past decades, but still to date, no clear consensus has been reached on the most optimal approaches to be utilized in the gut microbiota research (Lepage et al., 2013, Morgan & Huttenhower, 2014, Franzosa et al., 2014, Fournier et al., 2015, Lagier et al., 2016).

2.2.1 Traditional methods and 'culturomics'

Bacterial identification from stool samples has traditionally rested upon bacterial cultivation on selective culture media, morphological examination, and

biochemical testing. These methods are still useful when searching for individual pathogens, for example when identifying the causing agent for gastroenteritis. However, the traditional identification methods are not optimal for comprehensive gut microbiota analysis, as most intestinal bacteria are obligate anaerobes that require specific culture conditions and complex media to grow *in vitro* (reviewed in Rajilić-Stojanović et al., 2007). Furthermore, the nutritional and environmental requirements of all gut bacteria have not been determined to date, and thus the currently available sample collection and culture methods may not be adequate for successful cultivation of all bacterial species residing in the gut (Browne et al., 2016, Lagier et al., 2016). In addition, the culture-based methods are relatively time-consuming and laborious (Lagier et al., 2015a). Therefore, high-throughput culture-based gut microbiota profiling would require new advancements in sample collection, storage, and culture methods (Lagier et al., 2015b, Browne et al., 2016).

During the past decade, identification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has significantly speeded up the identification of the cultivated bacteria, as the use of MALDI-TOF mass spectrometry has suppressed the need for Gram staining and biochemical methods in the identification process (Lagier et al., 2016). Consequently, the culture-based bacterial isolation has become slightly more practicable, and Raoult and co-workers have introduced a concept of ‘culturomics’, a culture-based approach for gut microbiota composition analysis where diverse culture conditions are accompanied by bacterial identification with MALDI-TOF mass spectrophotometry and 16S rRNA gene sequencing (Lagier et al., 2012, Lagier et al., 2015a). Culturomics is a bacterial isolation strategy where the culture conditions are adjusted and diversified in order to mimic the natural conditions of the GI tract (Fournier et al., 2015, Lagier et al., 2015b). By utilizing an extremely wide variety of different culture conditions, over a thousand different prokaryotic species have already been isolated from the human gut, including several novel bacterial species (Lagier et al., 2016). Nevertheless, the culturomics approach is extremely labor-intensive and time-consuming; Raoult et al. have themselves reported that the identification of 1057 bacterial species has required cultivation and analysis of over 900 000 bacterial colonies (Lagier et al., 2016). Even though the tested culture conditions have been standardized and ranked (Lagier et al., 2016), sufficient mimicking of the gut habitat still requires an enormous variation of different culture conditions, thus demanding time and resources. Therefore, average microbiology laboratories are not easily adapted for the application of these methods.

2.2.2 Molecular methods

In the past decades, in both diagnostics and research, the traditional culture-based methods have for the most part been substituted, or at least complemented, by culture-independent molecular methods (Turnbaugh et al., 2007, Hamady & Knight, 2009, Costello et al., 2009). The molecular biology -based methods do not require viable bacteria, as the detection and quantification of the microbes is based on isolation and identification of the molecular fingerprints rather than viable organisms. The study of genetic material extracted directly from a defined environment or ecosystem is referred to as metagenomics, and the genetic catalog of the gut microbiota is called gut microbiome (Turnbaugh et al., 2007, Lepage et al., 2013). The molecular methods are often less time-consuming and more specific than the traditional cultivation-based methods in studying complex microbial communities, and consequently, metagenomics has become the method of choice for investigating the structure and functions of the gut microbiota (Lepage et al., 2013, Zhang et al., 2018). Further, as recent methodological improvements have enabled significant decreases in analysis costs, the basic molecular methods are no longer dramatically expensive as compared to the traditional methods. Metagenomics has vastly expanded the knowledge and understanding of the GI tract microbiome, and different molecular methods are increasingly used in studying the host-microbe interactions and the metabolic activities of the gut bacteria (Navas-Molina et al., 2013, Lepage et al., 2013, Kundu et al., 2017).

2.2.2.1 16S rRNA gene amplification and sequencing

16S rRNA, referring to the 16S small subunit ribosomal RNA, is a component of the small (30S) subunit of the prokaryotic ribosome (Stern et al., 1989). 16S rRNA encoding genes are present in all bacteria and archaea, but the copy number of the gene varies between organisms (Lee et al., 2008). The length of the microbial 16S rRNA gene in approximately 1500 bp. Certain regions of the gene are highly conserved between the microbial species and have an extremely slow evolutionary rate (Van de Peer et al., 1996). In addition to these highly conserved gene regions, the 16S rRNA gene generally includes nine hypervariable regions (V1-V9; Figure 2) that possess significant sequence dissimilarity between distinct microbial species (Van de Peer et al., 1996, Chakravorty et al., 2007). Due to these properties, the 16S rRNA gene serves as a valuable tool for phylogenetic and taxonomic analyses, along with species identification, of the micro-organisms (Van de Peer et al., 1996, Chakravorty et al., 2007, Zhang et al., 2018). In fact, 16S rRNA sequencing has been one of the

most commonly used methods in bacterial classification and species identification since the late 1990s. The length of each 16S rRNA gene hypervariable region is about 30 to 100 bp, being thus well suited for PCR amplification and other molecular biology techniques (Van de Peer et al., 1996). On the other hand, the degree of sequence diversity varies between the variable regions, and none of the hypervariable regions is alone adequate for species-specific identification of all bacteria (Chakravorty et al., 2007). Therefore, analysis of the full-length 16S rRNA gene is required for most comprehensive and reliable bacterial species identification (Chakravorty et al., 2007).

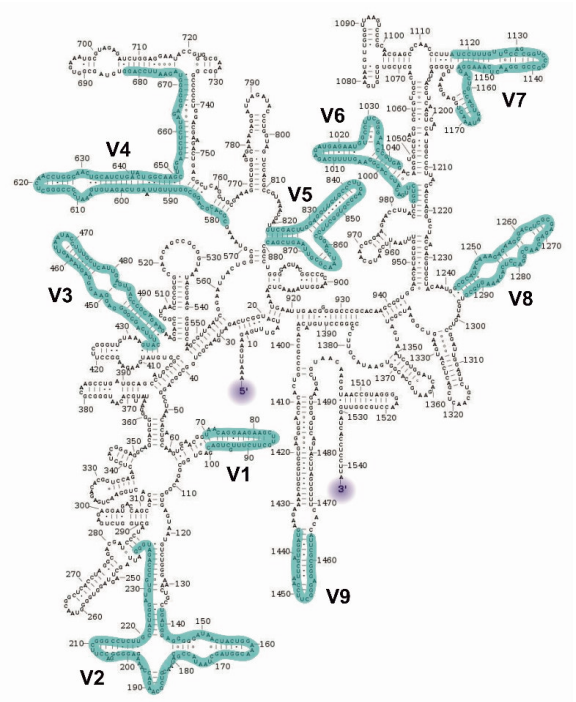

Figure 2. 16S rRNA of *Escherichia coli*. The 16S rRNA secondary structure has been adapted from XRNA gallery (http://rna.ucsc.edu/rnacenter/xrna/xrna_gallery.html). The hypervariable regions (V1-V9) are highlighted based on (Chakravorty et al., 2007).

One of the first 16S rRNA -targeting methods utilized in gut microbiota research was the amplification of the bacterial 16S rRNA encoding gene by polymerase chain reaction (PCR) using species/group specific primers (reviewed in Lepage et al., 2013). Real-time quantitative PCR (qPCR) with carefully designed primers enables both identification and quantification of the targeted bacterial species or groups, but the identification is restricted to the targeted bacteria while no information about the overall bacterial composition is obtained. Techniques that target the bacterial 16S rRNA gene and other genetic markers have, however, significantly proceeded during the past few decades. Especially the next

generation sequencing (NGS) of the 16S rRNA gene has become a universally accessible method for determining the 16S rRNA profiles of the GI tract microbiota. In next-generation 16S rRNA gene sequencing, one or several hypervariable regions of the bacterial 16S rRNA gene are amplified, and the sequences from each original sample are identified using sample-specific index sequences (Klindworth et al., 2013). Then, the 16S rRNA gene libraries are sequenced with a high-throughput instrument, e.g. Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) or Ion Torrent (Thermo Fischer Scientific, Waltham, MA, USA), and the sequence data analysis is performed with specific bioinformatic analysis tools. Briefly, the sequences are trimmed and clustered, and then compared to known sequences in reference databases, e.g. Greengenes (DeSantis et al., 2006) or SILVA (Quast et al., 2012). Amplification with universal primers, accompanied by NGS, enables fast and high-throughput analyses of complex microbial communities, while sample identification advancements and high-throughput sequencing instruments allow analyses of several specimens simultaneously. These methodological advancements have led to a significant decrease in the sequencing costs, making the 16S rRNA gene sequencing readily feasible in the gut microbiota research (Tringe & Hugenholtz, 2008, Hamady & Knight, 2009, Mandal et al., 2015). Ever since the introduction of the 16S rRNA gene targeting NGS, the knowledge on the composition of the gut microbiota has greatly expanded (Tringe & Hugenholtz, 2008, Costello et al., 2009, Qin et al., 2010).

2.2.2.2 Limitations of the 16S rRNA gene sequencing

While the 16S rRNA gene sequencing has become more cost-effective and thus more readily available, it has also become increasingly evident that the molecular methods have weaknesses; several studies have emphasized that all metagenomic analyses are prone to severe biases (Cai et al., 2013, Kennedy et al., 2014, Brooks et al., 2015). The first potential cause for bias in the 16S rRNA gene sequencing, as in all molecular techniques, is the sample collection and pre-processing. Sample material in gut microbiota studies usually consist of stool samples, rectal swabs, or endoscopic biopsies, and as the bacterial ecosystem in these samples is extremely complex, an inconsiderate sample collection or storage can have a significant impact on the study results (Salonen et al., 2010, Maukonen et al., 2012). For example, sample freezing has been reported to substantially affect the observed *Firmicutes*-to-*Bacteroidetes* ratio (Bahl et al., 2012). It is therefore outstandingly important that all samples in one study are collected and stored identically, applying methods that minimize the risk of alterations in the microbial composition prior to downstream analyses

(Maukonen et al., 2012). The second critical step in gut microbiota studies is the DNA extraction; DNA isolation method has been proposed to significantly affect the results of the NGS analyses (Maukonen et al., 2012, Wesolowska-Andersen et al., 2014, Burbach et al., 2016). For example, the ratio between observed Gram-positive and Gram-negative bacteria may vary significantly depending on the applied DNA extraction protocol, as the cell wall of Gram-positive bacteria is notably stronger and more difficult to lyse than the cell wall of Gram-negative bacteria (Costea et al., 2017). It has been generally acknowledged that rigorous lysis, e.g. adequate bead-beating, is required in order to successfully extract DNA from certain Gram-positive bacteria such as *Bifidobacterium* (Maukonen et al., 2012, Santiago et al., 2014, Walker et al., 2015).

In addition, the NGS results are highly dependent on the analysis methods themselves; for example, the targeted 16S rRNA gene region and the sequencing platform have been shown to significantly influence the outcome of the gut microbiota composition studies (Wang et al., 2007, Liu et al., 2008, Klindworth et al., 2013, Starke et al., 2014, Tremblay et al., 2015, Fouhy et al., 2016). And further, the sequence data analysis methods can affect the result output (Hamady & Knight, 2009, Bokulich et al., 2013, Weiss et al., 2017). And in addition to the method-dependent biases, another important frailty in the 16S rRNA gene sequencing based gut microbiota analyses is the limited sequencing depth of the current NGS techniques (Jovel et al., 2016). As molecular methods can identify bacterial species that are not cultivable *in vitro,* they are often considered extremely sensitive. It has, however, been reported that the existing 16S rRNA gene sequencing methods are actually not optimal for acknowledging bacteria at low concentrations, and because of this, several bacterial species/genera that present abundances below the detection threshold, yet still potentially possess clinical importance, may be missed (Lagier et al., 2012). Increased sequencing depth, on the other hand, predisposes to external contamination and sequencing artifacts (Jovel et al., 2016). Thus, research performed with the current 16S rRNA gene sequencing methodologies must compromise between the detection threshold and the risk of false findings among the detected taxa. For these reasons, reliable comparison of different 16S rRNA gene based NGS studies remains a challenge (Hamady & Knight, 2009).

16S rRNA gene sequencing based methods provide relative rather than absolute quantification of the bacteria, and even though certain predictive functional analysis packages are available (Langille et al., 2013, Aßhauer et al., 2015), no specific conclusions about the functions of the gut microbiota should be made solely based on 16S RNA gene profiles. In addition, due to limited sequencing length, differentiation of closely related bacterial species is deficient with the currently available 16S rRNA based NGS methods; bacteria from same genera

often obtain close to identical sequences across the region that can be sequenced at once (Chakravorty et al., 2007). Therefore, even though the 16S rRNA based NGS analysis is a valuable tool in gut microbiota composition analyses, its obvious weakness is the low taxonomic resolution (Jovel et al., 2016, Ranjan et al., 2016). There has recently been significant progress in the development of technologies providing longer sequencing reads, and these methods may provide a solution for this issue as they enable full-length 16S rRNA gene sequencing (Bleidorn, 2016, van Dijk et al., 2018). At present, the general use of these long-read sequencing technologies is limited due to high cost and relatively high sequencing error rates (Goodwin et al., 2016, Bleidorn, 2016). Consequently, in recent years, 16S rRNA gene sequencing has been increasingly substituted by more comprehensive analysis methods, mainly metagenomic shotgun sequencing (Table 2). However, as shotgun sequencing is noticeably more expensive and requires more hardware resources, 16S rRNA gene sequencing is still often the method of choice for the analysis of gut microbiota composition in large sample cohorts (Jovel et al., 2016).

2.2.2.3 Shotgun metagenome sequencing, shotgun metatranscriptome sequencing, metaproteomics, and metabolomics

In shotgun sequencing, instead of generating and sequencing amplicon libraries of 16S rRNA gene or some other specific gene target, the whole DNA or RNA (cDNA) content of the sample is sequenced in short reads and then analyzed based on comparison with sequence databases (reviewed in Lepage et al., 2013). DNA-targeting shotgun sequencing of a complex microbial ecosystem is referred to as shotgun metagenome sequencing. Shotgun metagenome sequencing can be used for bacterial taxonomic assignment, but also for screening certain genes and/or gene sets in a microbial ecosystem (Morgan & Huttenhower, 2014). It is able to provide information about e.g. metabolic and functional capability of the microbiome, which is an important advantage in comparison to the 16S rRNA based NGS that provides information merely on the bacterial composition and diversity (Table 2; Jovel et al., 2016). Consequently, shotgun metagenome sequencing is an increasingly popular method in the gut microbiota research. In addition to studying complex microbial ecosystems, the method can also be used for reconstructing whole genome sequences of single bacteria, being thus useful for analyzing bacterial genomes from pure cultures (Fleischmann et al., 1995).

As long as decent sequencing depth is acquired, shotgun metagenome sequencing provides significant information about the microbial composition and overall gene content of the bacterial ecosystems (Ranjan et al., 2016). Nevertheless, the

method is not able to provide information about the actual activity of the observed genes (Franzosa et al., 2014), and metatranscriptomic, metaproteomic, or metabolomic applications are required for genuine functional analyses (Table 2). Term 'metatranscriptomics' refers to the analysis of community-wide gene expression profiles of complex ecosystems (Bashiardes et al., 2016). While shotgun metagenome sequencing is targeting the ecosystem's whole DNA, in shotgun metatranscriptome sequencing, the messenger RNA (mRNA) content of the ecosystem is analyzed (Morgan & Huttenhower, 2014, Bashiardes et al., 2016).

Table 2. Culture-independent approaches to study the human gut microbiota

Approach	Target & outcome	Advantages and limitations
16S rRNA gene sequencing	Target: 16S rRNA gene Outcome: bacterial and archaeal composition and diversity	+ Least expensive of the molecular methods + High-throughput and relatively easy to perform + Extensive reference databases available – Provided information is limited to taxonomic composition and microbial diversity – Reliable taxonomic resolution is limited to genus level – Does not distinguish between dead and alive bacteria – Does not provide information about viruses or eukarya – Method-dependent biases and batch effects due to sample storage, DNA extraction, PCR, and sequencing
Shotgun metagenome sequencing	Target: genomic DNA Outcome: bacterial, archaeal, fungal, and viral composition and overall gene content	+ Enables bacterial species-level identification + Besides taxonomic analyses, provides information about the functional capacity of the microbes – More expensive and laborious than amplicon sequencing – Requires extensive hardware capacity – Prone to similar biases as 16S rRNA gene sequencing – Does not provide information about the activity of the observed genes
Shotgun meta-transcriptome sequencing	Target: cDNA prepared from microbial mRNA Outcome: bacterial, archaeal, fungal, and viral transcriptomes, i.e. gene expression	+ Provides information about the actual gene activity + Can be used for measuring functions and functional changes of the microbes under certain physiological / environmental conditions – Prone to biases caused by sample processing, as RNA is significantly less stable than DNA – mRNA represents only a minority of fecal RNA → rRNA and tRNA need to be removed before reverse transcription – Prone to host mRNA contamination – Requires extensive hardware capacity – More expensive and laborious than shotgun metagenome sequencing
Meta-proteomics	Target: proteins / peptides Outcome: intestinal protein profile	+ Has potential to provide a view of microbial gene activity under certain psychological / environmental conditions – No standardized protocols available – Exclusion of host- or diet-derived proteins not feasible – Reference databases still incomplete – Requires extensive hardware capacity
Metabolomics	Target: metabolites Outcome: profile of the end products of microbial and host metabolism	+ Can be performed as targeted or untargeted analyses + Has potential to provide means for e.g. disease biomarker discoveries – No standardized protocols available – Discrimination between microbial and host metabolites not feasible – Reference databases still incomplete – Requires extensive hardware capacity

Shotgun metatranscriptome sequencing can provide information about the functions and functional changes of the intestinal ecosystem under certain conditions (Morgan & Huttenhower, 2014, Franzosa et al., 2014), and the method holds great promise in enhancing the understanding of the microbial functions and host-microbe interactions in different health-related conditions (Bashiardes et al., 2016). Consequently, shotgun metatranscriptome sequencing is increasingly used in gut microbiota research. However, the method holds several challenges that need to be acknowledged in the technical setup (Table 2), and besides making the method expensive and time-consuming, these special requirements make shotgun metatranscriptome sequencing extremely prone to biases. Therefore, the full potential of the methodology is yet to be harnessed in gut microbiome research (Morgan & Huttenhower, 2014).

While metatranscriptomics examine the gene activity of the microbial ecosystem, metaproteomics and metabolomics are intended for providing information about the microbiome's molecular activities (Table 2; Morgan & Huttenhower, 2014). Basically, gut metaproteomics refers to mapping and profiling of microbial proteins (reviewed in Petriz & Franco, 2017), while gut metabolomics refers to profiling of microbial, along with host, metabolites (reviewed in Vernocchi et al., 2016). These methods hold promise, for example, in determining differences in protein production under certain physiological conditions. The methods are still in their infancy regarding the gut microbiome research, and no standardized protocols for the proteomic or metabolomics analyses of the intestinal microbes currently exist (Morgan & Huttenhower, 2014, Petriz & Franco, 2017). Nevertheless, both methods indisputably have great potential to provide valuable information concerning the biology of the intestinal microbial ecosystem, and methodological development is determinedly ongoing (Wilmes et al., 2015, Vernocchi et al., 2016, Petriz & Franco, 2017).

2.2.3 Future aspects in gut microbiota research

At the moment, the method development in the gut microbiota research field is extremely rapid. For example, several so-called third-generation sequencing protocols, enabling longer sequencing read lengths, are under constant development, and these methods are expected to provide a solution for some of the current weaknesses in the taxonomic analyses of 16S rRNA gene sequencing and shotgun sequencing (Bleidorn, 2016, van Dijk et al., 2018). Meanwhile, there is constant methodological progress in the metatranscriptomic, metaproteomic, and metabolomic approaches (Wilmes et al., 2015, Bashiardes et al., 2016, Matysik et al., 2016). And finally, the development of bioinformatic analysis

tools for gut microbiome research is continuous. Several open source software packages are already available for basic microbiome data analysis (Schloss et al., 2009, Caporaso et al., 2010), but only few of the existing tools are adequately user-friendly for basic scientists with limited bioinformatic knowledge (Dhariwal et al., 2017). Further, in order to obtain a comprehensive view of the gut microbiota composition and functions, the taxonomic data, i.e. the 16S rRNA gene sequencing or shotgun metagenome sequencing data, should ideally be integrated with the functional data, i.e. the metatranscriptomic, metaproteomic, and/or metabolomic data, and this data integration requires the development of novel data analysis methods (Morgan & Huttenhower, 2014, Franzosa et al., 2014). In fact, as the modern data analysis methods that allow adequate integration of the diverse meta-omics data are likely to require both extensive computational power and advanced bioinformatic tools, the future of gut microbiota research appears to largely lean on competent bioinformatic specialists (Franzosa et al., 2014, Jovel et al., 2016).

While the molecular methods unquestionably have the potential to provide high-resolution picture of the gut microbiota composition and the host-bacteria interactions, the culturomics strategy (discussed in Chapter 2.2.1) has also offered a great deal of new information concerning the prokaryotic inhabitants of the human gut (Sankar et al., 2015, Lagier et al., 2016). In fact, this so called renaissance of the bacterial culture has, to some extent, challenged the molecular biology methods in gut microbiota analysis, as in certain cases, culturomics seem to produce more comprehensive results than the metagenomic approaches (Dubourg et al., 2013, Lagier et al., 2015a). Further, it has been reported that the culture-based and molecular-based gut microbiota composition analyses tend to result in significantly divergent outcomes; it has been stated that only a minority of the bacterial species present in a human fecal sample can be detected with both culturomics and metagenomics (Lagier et al., 2012). Therefore, if method development led to further reduction in the required culture conditions, culturomics could provide a useful supplement to the molecular microbiology methods in the gut microbiota research and especially in the bacterial taxonomy assignment (Fournier et al., 2015, Sankar et al., 2015). Bacterial cultivation is still required for physiological characterization of the bacteria, and further, the molecular methods have not yet been able to replace culture-based methods in antibiotic susceptibility testing. It therefore appears that both bacterial cultivation and meta-omics are required for comprehensive understanding of the gut microbiota composition and function (Lagier et al., 2015a).

2.3 Obesity, metabolic disorders, and gut microbiota

The worldwide prevalence of obesity has almost tripled since 1975 (World Health Organization, 2017). WHO has estimated that, in 2016, more than 1.9 billion adults were overweight (BMI ≥ 25 kg/m^2), and of these, over 650 million were obese (BMI ≥ 30 kg/m^2). Overall, in 2016, approximately 13 % of the World's adult population were obese. Obesity is generally defined as excessive fat accumulation that is a consequence of excess energy intake in comparison to energy consumption. Mechanistically, however, obesity is a complex metabolic disorder that often disrupts the overall metabolism of the body (Arora et al., 2013). The excess energy intake leads to an induction of several metabolic cascades that aim at balancing the energy disequilibrium of the body. Thus, as a result of the increased prevalence of obesity, the burden of obesity-related metabolic disorders like T2D and NAFLD has also expanded. Clustering of several obesity-related conditions can be referred to as 'metabolic syndrome', which is a comprehensive disorder in energy distribution and storage (Kaur, 2014). Visceral adiposity, IR, elevated blood pressure, and hyperglycemia are some of the several constituting factors of the syndrome. Metabolic syndrome significantly increases the risk of cardiovascular diseases and T2D (Kaur, 2014). On the other hand, all obese individuals do not develop metabolic syndrome.

2.3.1 Insulin resistance

Insulin is an important anabolic hormone that regulates the metabolism of carbohydrates, fats, and proteins. The main physiological stimulus for insulin synthesis is glucose; in a normal situation, body responses to high blood glucose level by inducing insulin secretion (Nelson & Cox, 2008). Insulin has several physiological effects, the most prominent being the induction of glucose intake by insulin sensitive organs such as adipose tissue and skeletal muscles. In the adipose tissue, insulin also plays an important role in maintaining a balance between fatty acid storage and the release of free fatty acids (FFAs) into the circulation, as insulin promotes triacylglycerol (TAG) synthesis and inhibits lipolysis, i.e. the breakdown of the lipids (Dimitriadis et al., 2011). In a normal physiological condition, adipose tissue is extremely sensitive to the blood insulin concentrations, whereas in obesity, the sensitivity of adipose tissue to respond to the blood insulin level is often decreased (Morigny et al., 2016). This state is called insulin resistance (IR), which is a condition that has been recognized in all insulin sensitive organs including adipose tissue, skeletal muscles, and liver (Nelson & Cox, 2008). IR predisposes the host to hyperglycemia, as the blood glucose is not normally absorbed by the cells. Subsequently, the insulin

production may also increase, resulting in blood hyperinsulinemia (Nelson & Cox, 2008). In general, IR is likely to affect the overall metabolism of the host. IR has been closely related to metabolic syndrome, and it is likely to contribute to the development of T2D (Nelson & Cox, 2008). The pathophysiology of IR during obesity, however, remains largely unknown. As well as obesity and T2D, IR has been strongly associated with the Western diet and sedentary lifestyle, and the condition is primarily treated by exercise and weight management (Kaur, 2014).

2.3.2 Non-alcoholic fatty liver disease

Obesity is often, yet not always, accompanied by excess liver fat accumulation. NAFLD is a condition where more than 5 % of liver cells present steatosis, i.e. accumulation of TAGs, without excessive alcohol consumption (Marchesini et al., 2016). In practice, NAFLD represents a wide spectrum of conditions from relatively mild steatosis to severe non-alcoholic steatohepatitis (NASH). NAFLD is an increasingly common health condition, and currently the most prevalent liver disorder in the Western countries (Adams & Lindor, 2007). The disorder is already affecting 20-30 % of general population and up to 75-100 % of morbidly obese individuals, and the prevalence is continuously increasing (Henao-Mejia et al., 2013).

The pathophysiology of NAFLD has not been fully elucidated, but the condition is strongly linked to obesity, metabolic syndrome, and IR (Adams & Lindor, 2007, Lomonaco et al., 2012, Bril et al., 2017). Metabolically healthy obese individuals rarely have hepatic steatosis, which suggests that metabolic stress is likely to play an important role in the development of NAFLD. One possible reason for the metabolic stress is hepatic lipotoxicity, which refers to intracellular accumulation of harmful lipid products (Cusi, 2010, Lomonaco et al., 2012). Consequently, adipose tissue dysfunction and IR are proposed to play important roles in the development of lipotoxicity and hepatic steatosis in NAFLD (Lomonaco et al., 2012, Bril et al., 2017). Adipose tissue is the primary source of FFAs for hepatic TAG synthesis, and adipose tissue IR is suggested to result in excess TAG lipolysis that subsequently causes an oversupply of the FFAs into the liver. The excess FFA supply may then cause excess hepatic TAG synthesis and lipotoxicity, which leads to further impairment in the insulin signaling, along with activation of different inflammatory pathways (Cusi, 2010, Lomonaco et al., 2012).

NAFLD can be diagnosed by imaging, serum biomarkers, and histology. Imaging is currently preferred as the first-line diagnostic tool due to its noninvasive nature

(Marchesini et al., 2016). In ultrasonography, the hepatic steatosis is detected as an increase in echogenicity (Joseph et al., 1991). The method is reliable in detecting moderate to severe steatosis, but it has limited sensitivity in detecting mild steatosis. In addition, the method is not optimal for examining patients with high BMI (Fishbein et al., 2005, Adams & Lindor, 2007, Marchesini et al., 2016). Further, ultrasonography is not reliable in the evaluation of the degree of steatosis, as the visual assessment is prone to significant inter-observer variability (Cengiz et al., 2014). Magnetic resonance imaging, by contrast, allows accurate quantitation of the liver fat content (Longo et al., 1995, Fishbein et al., 2005), but this method is not optimal for general screening in clinical practice due to relatively high cost and long imaging time (Marchesini et al., 2016). Mild to moderate elevation in serum AST, ALT, or both, is common in NAFLD patients, and often the only observed laboratory finding. Several serum biomarkers and index scores have been generated for predicting the presence and severity of NAFLD (Marchesini et al., 2016), but the validity of these indicators in disease diagnostics remains unclear (Sberna et al., 2018). Histology is still the best diagnostic tool for analyzing the severity of NAFLD, and liver biopsy is required in order to differentiate hepatic steatosis from NAFLD's more severe form, NASH (Marchesini et al., 2016).

Hepatic steatosis is usually a reversible condition that can be treated by lifestyle changes, i.e. weight loss via healthier diet and increased exercise (Lazo et al., 2010, Jordy et al., 2015). Several agents, such as vitamin E and certain diabetes medications, have been tested in the treatment of NAFLD, but the results have been controversial (Marchesini et al., 2016). Therefore, in addition to lifestyle improvements, no approved prevention or treatment strategies for NAFLD currently exist. This is a problem in the developed countries, where people are accustomed to their unhealthy lifestyle including high-calorie diet and lack of exercise. In addition, most NAFLD patients are symptomless (Adams & Lindor, 2007), which is likely to further decrease the motivation for lifestyle changes. As untreated NAFLD can progress into NASH, liver cirrhosis, or even hepatocellular carcinoma (Adams & Lindor, 2007), investigation of novel prevention and treatment strategies for the disease is increasingly important.

2.3.3 Gut microbes in obesity and metabolic disorders

A causal link between gut microbes and host metabolism was first proposed already over a decade ago, when mouse studies demonstrated that fecal microbiota transplantation from obese mice was able to replicate the obese phenotype in GF mice (Bäckhed et al., 2004, Turnbaugh et al., 2008). This

phenomenon may to be linked to the ability of gut microbes to process otherwise indigestible dietary nutrients and, consequently, increased deposition of extracted energy (Bäckhed et al., 2004). Accordingly, in both humans and mice, the gut microbiota of obese individuals has been shown to differ from the microbiota of lean individuals (Ley, 2010, Gérard, 2016). The reports regarding the gut microbiota composition in obesity have, however, been rather inconsistent. For example, findings regarding the abundance of phyla *Firmicutes* and *Bacteroidetes* have been highly discordant (Bäckhed et al., 2004, Ley et al., 2005, Duncan et al., 2008, Schwiertz et al., 2010, Raman et al., 2013).

Obesity is often associated with low-grade inflammation, which has been suggested to further contribute to IR, T2D and other metabolic disorders (Makki et al., 2013, Piya et al., 2013). Mouse studies have demonstrated that diet-induced obesity, i.e. obesity developed in response to HFD, is associated with an increased secretion of pro-inflammatory cytokines (Xu et al., 2003, Ding et al., 2010). Microbial endotoxins have been put forward as one possible underlying stimuli for the immunological functions and metabolic rearrangements associated with obesity; endotoxins, such as lipopolysaccharides (LPS), may possess capability to induce inflammation and adverse metabolic effects on the host (Cani et al., 2007, Kopp et al., 2009, Könner & Brüning, 2011). LPS is the major component of the outer membrane of Gram-negative bacteria and known to cause inflammation when ending up in the circulation (Beutler & Rietschel, 2003, Brun et al., 2007, Amar et al., 2011). The main pattern recognition receptor for LPS is Toll-like receptor 4 (TLR4) that, likely as a complex with MD-2, is present on the surface of several immune cells and various other cell types, including adipocytes and hepatocytes (Lu et al., 2008, Boutagy et al., 2016). The recognition of LPS by TLR4 initiates an activation of multiple signaling components, such as NF-κB, and subsequent production of pro-inflammatory cytokines that further activate the innate immune system (Cani et al., 2007, Lu et al., 2008, Boutagy et al., 2016). The binding of LPS to the TLR4-MD-2 complex is preceded by an activation of a co-receptor CD14, to which LPS is presented by LPS-binding protein (Lu et al., 2008). CD14 is present on the membrane of several TLR4-presenting cells, but also in a soluble form (Lu et al., 2008).

In mice, increased energy intake, e.g. due to HFD, is suggested to increase gut permeability and to cause systemic, low-level increase in the plasma LPS levels (Cani et al., 2007, Brun et al., 2007). While severe systemic infections often cause over a hundred-fold increase in the circulating endotoxin concentration compared to healthy individuals (Opal et al., 1999), the endotoxemia level induced by HFD is considerably lower; in mice, HFD has been demonstrated to induce approximately 2- to 4-fold increase in the concentration of circulating LPS (Cani et al., 2007, Brun et al., 2007). This low-grade elevation in the plasma

LPS level has been hypothesized to serve as a possible mediator of metabolic imbalance observed in obesity, as it has been associated with elevated body weight, adipose tissue and liver weight, increased pro-inflammatory cytokine levels, and fasted hyperglycemia and insulinemia (Cani et al., 2007, Brun et al., 2007). Due to the possible link between the low-level endotoxemia and metabolic disorders, the condition has been termed 'metabolic endotoxemia' (Cani et al., 2007). Recently, gut microbiota dysbiosis has been strongly linked to gut permeability and obesity-associated low-grade inflammation (Sekirov et al., 2010, Modi et al., 2014, Graf et al., 2015, Becker et al., 2015). Therefore, it seems evident that certain gut microbes - especially the endotoxin-possessing bacteria - could induce the metabolic endotoxemia and, consequently, contribute to the development of obesity and obesity-linked metabolic diseases (Cani et al., 2007, Sekirov et al., 2010). This hypothesis is reinforced by the fact that GF mice are immune to the diet-induced obesity and IR (Bäckhed et al., 2007, Ding et al., 2010). Profound studies are, however, needed in order to elucidate the mechanism underlying the initiation of the obesity-associated inflammatory cascades.

In addition to obesity and IR, NAFLD has also been associated with intestinal dysbiosis and the abundance of certain gut microbes (Miele et al., 2009, Zhu et al., 2013, Raman et al., 2013, Munukka et al., 2014, Boursier et al., 2016, Shen et al., 2017). For example, subjects with NAFLD have been reported to obtain increased gut permeability (Miele et al., 2009) and elevated abundance of intestinal *Enterobacteriaceae* (Zhu et al., 2013, Özkul et al., 2017, Shen et al., 2017). On the other hand, low *F. prausnitzii* (Munukka et al., 2014) and *Akkermansia muciniphila* (Özkul et al., 2017) abundances have been reported in NAFLD patients compared to healthy individuals. Both *F. prausnitzii* and *A. muciniphila* are believed to support epithelial integrity and gut health (Martin et al., 2014, Schneeberger et al., 2015, Reunanen et al., 2015), whereas certain *Enterobacteriaceae* genera have been associated with metabolic endotoxemia (Yan et al., 2016). Meanwhile in mice, gut microbiota composition has been demonstrated to contribute to liver fat accumulation even independently of diet and obesity (Le Roy et al., 2013). As the GI tract is connected to the liver via hepatic portal system, liver is well exposed to the intestinal molecules and metabolites (Abu-Shanab & Quigley, 2010). Therefore, in the case of leaky gut, i.e. increased gut permeability, the translocated bacteria, bacterial endotoxins, and other molecules originating from the gut may be capable of directly inducing the hepatic fat accumulation (Figure 3; Henao-Mejia et al., 2013, Leung et al., 2016, Tilg et al., 2016). On the other hand, results of some studies propose that adipose tissue inflammation may be the link between the intestinal dysbiosis and NAFLD (Munukka et al., 2014, Pekkala et al., 2015). The underlying mechanisms, however, remain to be determined.

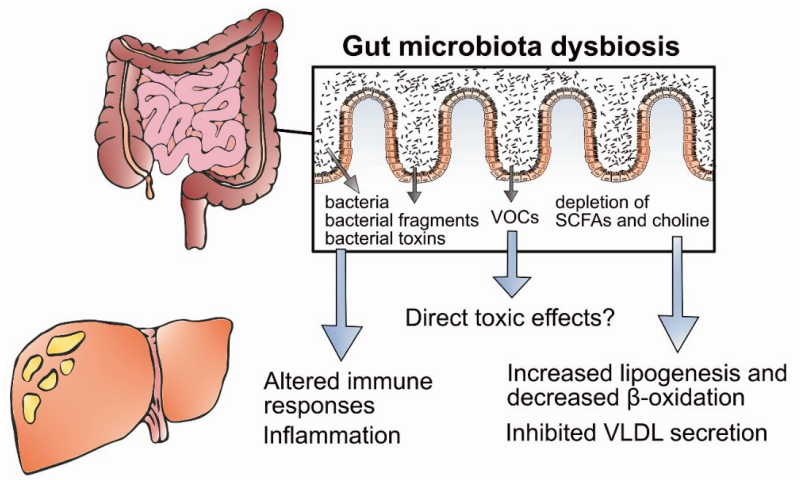

Figure 3. Gut microbiota dysbiosis -related mechanisms that may contribute to hepatic fat accumulation. Gut-liver axis is proposed to play a role in the progression of hepatic steatosis and non-alcoholic fatty liver disease (NAFLD). Gut microbiota dysbiosis disrupts the gut barrier function and leads to an increased gut permeability, i.e. leaky gut, which allows an excess flux of bacteria, bacterial toxins, and bacterial fragments (e.g. endotoxins) from the gut lumen (Leung et al., 2016). This causes metabolic endotoxemia and immune dysfunction that can induce liver inflammation and NAFLD. Meanwhile, dysbiotic gut microbiota typically represents decreased short-chain fatty acid (SCFA) production due to low abundances of *Firmicutes*. As SCFAs regulate hepatic lipid metabolism, SCFA depletion may cause excess hepatic lipogenesis and reduced β-oxidation that increase the accumulation of free fatty acid in liver (Tilg et al., 2016). In addition, intestinal dysbiosis is suggested to associate with reduced choline availability, which can cause decreased synthesis and secretion of VLDLs from the liver and thus further induce the hepatic steatosis (Abu-Shanab & Quigley, 2010). Gut microbiota dysbiosis can also increase the endogenous production of volatile organic compounds (VOCs) such as ethanol due to increased abundances of *Proteobacteria* (Leung et al., 2016). VOCs may contribute to liver injury and NAFLD progression by further increasing the intestinal permeability and consequently triggering the metabolic endotoxemia. In addition, VOCs may have direct toxic effects on the liver (Leung et al., 2016). Proposedly several of these mechanisms are involved in the progression of NAFLD, but the pathways may vary between individuals.

2.3.4 Methods to study the role of gut microbes in obesity and metabolic disorders

The first groundbreaking findings regarding the possible causality between gut microbiota and obesity were drawn from studies where initially GF mice were colonized with gut microbiota from conventionally bred mice (Bäckhed et al., 2007, Turnbaugh et al., 2008). In these studies, as already mentioned above, GF mice were shown to be resistant to diet-induced obesity, whereas colonization with gut microbiota resulted in significant weight gain (Bäckhed et al., 2007, Turnbaugh et al., 2008). Since then, GF rodents have been extensively used in studies concerning the role of gut microbiota in obesity and metabolic disorders. GF rodents serve as a simple model to study the effects of individual bacterial species on immune functions and metabolism, and indeed, colonization of GF rodents with certain gut bacterial species has been shown to cause significant changes in the gut barrier function (Wrzosek et al., 2013) and on overall metabolism (Bäckhed et al., 2007, Fei & Zhao, 2013).

As the GF rodents completely lack the normal microbiota and thus possess altered immunological functions (Al-Asmakh & Zadjali, 2015), these artificial models are not quite optimal for profound studies concerning the role of gut microbiota in the onset of metabolic disorders. In addition, intestinal monocolonization of single bacterial species is likely to have distorted effects on GF mice, because the microbial inter-species networks are completely missing. Consequently, several recent studies investigating the role of gut microbes in obesity and metabolic disorders have been performed with conventionally bred specific-pathogen-free (SPF) rodents that possess diverse gut microbiotas and functional immune systems (Brun et al., 2007, Podrini et al., 2013, Jordy et al., 2015, Xie et al., 2016). In these otherwise metabolically healthy mice, the metabolic disturbances are usually induced by HFD-feeding. For example, C57BL/6 and ICR mice have been shown to develop obesity and NAFLD in response HFD (Podrini et al., 2013, Xin et al., 2014), and as a result, these mice are regularly used for studying diet-induced obesity and metabolic disorders. Studies on HFD-fed mice have shown that administration of specific bacterial strains can modify the metabolic and immunological functions of the host. For example, intragastric administration of certain *Bifidobacterium* or *Lactobacillus* stains have been demonstrated to reduce liver steatosis in HFD-fed mice (Cano et al., 2013, Xin et al., 2014), suggesting that gut microbiota modulation may have potential to prevent diet-induced NAFLD. Some studies, however, have reported significant inter-individual variation in the susceptibility of the mice to HFD-induced metabolic disorders, suggesting that like humans, each SPF rodent may harbor a unique gut microbiota (Le Roy et al., 2013). This highlights the need for

adequate pilot studies and standardization in order to produce high quality interventions with minimal within-group variation.

In addition to dietary models, several animal models that genetically possess metabolic disturbances have been generated. One generally approved model is *ob/ob* mouse, which possesses a spontaneous mutation in leptin gene and, in the absence of proper leptin-regulated control of satiety, eats excessively (Coleman & Hummel, 1973). In comparison to wild type mice, *ob/ob* mice are extremely obese and possess hyperglycemia, IR, and hepatic steatosis (Coleman & Hummel, 1973, Takahashi et al., 2012). Another useful model is *db/db* mouse, which has a natural mutation in the leptin receptor gene and consequently develops nearly identical metabolic disturbances as the *ob/ob* mice (Chen et al., 1996). Both *ob/ob* and *db/db* mice are especially used in T2D research, yet their phenotype makes them suitable for studying several aspects of the metabolic syndrome (Takahashi et al., 2012).

None of the present animal models completely reflects to the human metabolic syndrome or to the hepatic pathophysiology of human NAFLD (Takahashi et al., 2012, Al-Asmakh & Zadjali, 2015). They have, however, revealed several mechanisms that potentially explain the metabolic disturbances associated with obesity and metabolic syndrome. For instance, several mouse studies have proposed that certain probiotics, i.e. live micro-organisms that have health-beneficial effects, may ameliorate obesity-related metabolic and immunological disturbances of the host (Kang et al., 2010, Cano et al., 2013, Xin et al., 2014, Ritze et al., 2014, Alard et al., 2016). These results have had an important role in boosting the design of corresponding human interventions investigating the possible effects of probiotics on human metabolism. Even though the information obtained from mouse studies cannot be directly generalized into humans (Al-Asmakh & Zadjali, 2015), some randomized controlled trials have successfully demonstrated the beneficial effects of certain probiotics also on humans (Alisi et al., 2014, Minami et al., 2015). These promising results corroborate that mouse models provide a valuable experimental tool for studying the host-microbe interactions associated with human diseases and disorders (Al-Asmakh & Zadjali, 2015).

2.4 Targeted gut microbiota modulation

Changing dietary habits is indisputably the most natural and effective way to modify both the gut microbiota composition and the metabolic health of an individual (Ravussin et al., 2012, Xiao et al., 2014, Graf et al., 2015, Ussar et al., 2015). Besides diet, several additional means for gut microbiota modification are

available, and constant research is carried out in order to clarify whether these approaches could be utilized in improving the metabolic health in a personalized manner.

2.4.1 Probiotics

Currently, one of the most common ways to modulate the gut microbiota composition is the consumption of probiotics and prebiotics. As mentioned above, probiotics are live micro-organisms that confer a health benefit on the host, whereas prebiotics are non-viable substrates that are selectively utilized by micro-organisms that confer beneficial health effects (Hill et al., 2014, Gibson et al., 2017). Most pro- and prebiotics are dietary supplements intended to support the GI tract homeostasis, and they are widely used for supporting the gut microbiota especially during antibiotic courses and dietary changes (Ohland & Macnaughton, 2010). The beneficial effects of the products are suggested to originate mainly from the enhanced intestinal barrier function that results from favorable immunomodulation and competitive colonization (Ohland & Macnaughton, 2010). The most commonly used bacterial probiotics belong to *Bifidobacterium* and *Lactobacillus* genera (Preidis & Versalovic, 2009, Arora et al., 2013). In addition, other lactic acid bacteria such as *Lactococcus* and *Streptococcus*, along with yeast *Saccharomyces*, are frequently used in probiotic supplements (Preidis & Versalovic, 2009, Arora et al., 2013). Additionally, genera *Bacillus*, *Enterococcus*, *Escherichia*, and *Propionibacterium* also include certain promising candidates for probiotic applications (Preidis & Versalovic, 2009). The most studied prebiotics, on the other hand, include polysaccharides like inulin and certain fructo-oligosaccharides that enhance the growth of *Lactobacillae* and *Bifidobacteria* (reviewed in Gérard, 2016).

Probiotics are suggested to prevent antibiotic-associated diarrhea (Sazawal et al., 2006, Preidis & Versalovic, 2009), and it is under constant research whether they could also ameliorate IBS and IBD (Preidis & Versalovic, 2009, McKenzie et al., 2016). Recently, both probiotics and prebiotics have also been suggested to possess anti-obesity properties (Lee et al., 2007, Kang et al., 2010, Gérard, 2016, Borgeraas et al., 2018). Together with reports suggesting that gut microbes can contribute to body weight accumulation (Bäckhed et al., 2004, Ley, 2010, Fei & Zhao, 2013), these findings indicate that the manipulation of intestinal microbial communities could serve as a possible future treatment strategy against obesity and metabolic disorders. The studies concerning the effects of probiotics on obesity have, however, produced partly controversial results, and the anti-obesity effects of probiotics have been demonstrated to be strictly strain-specific. For

example, some traditional probiotics like *L. acidophilus* have been shown to rather promote weight gain than to induce weight loss (reviewed in Million et al., 2012).

There has lately been increased interest in utilizing certain commensal gut microbes as novel probiotic agents in order to improve human health. These new probiotic candidates, referred to as next-generation probiotics, include potentially health-promoting commensal bacteria such as *F. prausnitzii* (Martin et al., 2017), *A. muciniphila* (Cani & Van Hul, 2015), and certain species from genera *Bacteroides* and *Clostridium* (Saarela, 2018). These candidate species possess different health-beneficial characteristics, and mouse studies have reported promising results regarding their capability to improve host health (Carlsson et al., 2013, Cani & Van Hul, 2015, Martin et al., 2015, Schneeberger et al., 2015, Martin et al., 2017, Saarela, 2018). Furthermore, the abundances of these bacteria have been associated with human health (Miquel et al., 2013, Stenman et al., 2015, Lopez-Siles et al., 2016). The knowledge on the efficacy and safety of these novel probiotic candidates in human consumption is still very limited, and comprehensive animal and human trials are needed in order to characterize the full probiotic potential of these bacteria (Saarela, 2018). For example, certain next-generation probiotic candidates such as *B. fragilis* are known to possess pathogenic potential, and thus careful safety assessments are crucial when evaluating the suitability of these bacteria for human consumption (Saarela, 2018). On the other hand, strain-specific differences in the metabolic capability of the commensal gut bacteria are only partially characterized (Martin et al., 2017), and additionally, various technological challenges must be met before strict anaerobes such as. *F. prausnitzii* can be commercially produced as probiotic supplements (Saarela, 2018). Even with these challenges the new probiotic candidates hold great promise, and gut microbiota modification by next generation probiotics may well provide a future approach for improving human metabolic health (Cani & Van Hul, 2015). It must, however, be acknowledged that the effects of pre- and probiotics are generally highly individual, and some people do not benefit from the products (Reid et al., 2010, Zmora et al., 2018). The differences in response to the products are likely to result, at least partly, from the differences in the indigenous gut microbiota composition between the individuals (Reid et al., 2010, Suez et al., 2018).

2.4.2 Fecal microbiota transplantation

Besides pre- and probiotics, other applications for gut microbiota modulation also exist. One of these applications is fecal microbiota transplantation (FMT),

where feces from a healthy donor in infused to the GI tract of a recipient (Cammarota et al., 2017). FMT is already a common practice for treating severe recurrent *C. difficile* diarrhea, and the treatment has been proven to be successful for at least approximately 90% of the *C. difficile* patients (Mattila et al., 2012, Kassam et al., 2013). FMT has been reported to induce long-term chances in the gut microbiota of the *C. difficile* patients (Jalanka et al., 2016), suggesting that the method could also have potential in treating other microbiota-associated diseases such as IBD (Colman & Rubin, 2014). Besides intestinal inflammation, the potential of FMT has also been studied in treating obesity and metabolic disorders (Gérard, 2016). As already mentioned, metabolic phenotypes have been successfully transmitted from conventionally bred mice to GF mice (Turnbaugh et al., 2006). In humans, allogenic gut microbiota infusion from lean donors has been shown to increase insulin sensitivity in subjects with metabolic syndrome (Vrieze et al., 2012). Nevertheless, the reports regarding the effects of donor BMI on the weight development of the FMT recipient are scarce and partly controversial (Alang & Kelly, 2015, Fischer et al., 2018). Therefore, even if FMT may have potential to serve as a possible future method in improving obesity and obesity-related metabolic disorders, it is not without concerns, and special care should be taken in the screening and selection of the donors. Altogether, well-designed clinical trials are required to unveil the full potential of FMT on improving the metabolic health of humans.

3 AIMS OF THE STUDY

The aim of this study was to evaluate the role of gut microbes in the emergence of metabolic disorders by studying the influence of targeted gut microbiota modulation on the metabolic status of the host.

The specific aims of this study:

- To set up a high-throughput molecular pipeline for gut microbiota composition analysis at the Institute of Biomedicine, University of Turku.
- To characterize the health effects of targeted gut microbiota modification by intragastric administration of *Faecalibacterium prausnitzii*, a bacterium previously linked to positive health outcomes.
- To study the effects of targeted gut microbiota modification by intragastric administration of *Enterobacter cloacae*, a flagella-bearing and endotoxin producing bacterium previously linked to obesity and metabolic disorders.

4 MATERIALS AND METHODS

4.1 Experimental design of the methodological study (I)

In this study, five commercial DNA extraction kits were tested and compared in order to establish an efficient and practical sample processing protocol to precede high-throughput gut microbiota analyses. Fresh stool specimens from four healthy Finnish adult donors were derived from a human study approved by the Ethics Committee of the Central Finland Health Care District. The performance of the DNA extraction kits was evaluated and compared based on ease of use, DNA gain, and diversity indices identified by the NGS. In addition, to evaluate the influence of the 16S rRNA gene sequencing protocol on the gut microbiota composition analysis results, two distinct 16S rRNA gene library preparation protocols were applied and the obtained diversity indices and the identified phylogenetic composition were compared. Further, the effect of a 21-day sample freezer storage (-75°C) on the 16S rRNA gene sequencing results was analyzed by comparing the DNA gain, identified bacterial profiles, and the diversity indices between the fresh and frozen specimens (unpublished results). Figure 4, on the next page, represents the structured scheme of the study.

4.1.1 DNA extraction from human fecal samples

Each stool specimen was homogenized by manual mixing and weighted into adequate subsamples according to the recommendations of the manufacturers of the five DNA extraction protocols. The DNA extractions were performed on the day of the sample collection for the fresh specimens and repeated after the 21-day freezer storage (-75°C). DNA extractions with MagNA Pure 96 DNA and Viral NA Large Volume Kit (MP; Roche Diagnostics GmbH, Mannheim, Germany) were performed only for the frozen stool specimens. For both fresh and frozen specimens, two parallel extractions were performed with each protocol. In GXT Stool Extraction Kit VER 2.0 (GXT; Hain Lifescience GmbH, Nehren, Germany), cell lysis was enhanced by an additional bead-beating with MOBIO PowerLyzer 24 Bench Top Bead-Based Homogenizer (MO BIO laboratories, Inc, Carlsbad, CA, USA). Otherwise, all the DNA extractions were performed by strictly following the manufacturers' protocols. The elution volume was 200 µl for GXT, QIAamp DNA Stool Mini Kit (QIA; QIAGEN GmbH, Hilden, Germany), and QIAamp Fast DNA Stool Mini Kit (QIAF; QIAGEN GmbH, Hilden, Germany), and 100 µl for MP and PowerFecal DNA Isolation Kit (MOB; MO BIO Laboratories, Inc., Carlsbad, CA, USA). The DNA

concentrations were measured with Qubit dsDNA HS assay kit and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and the DNAs were stored at -75°C.

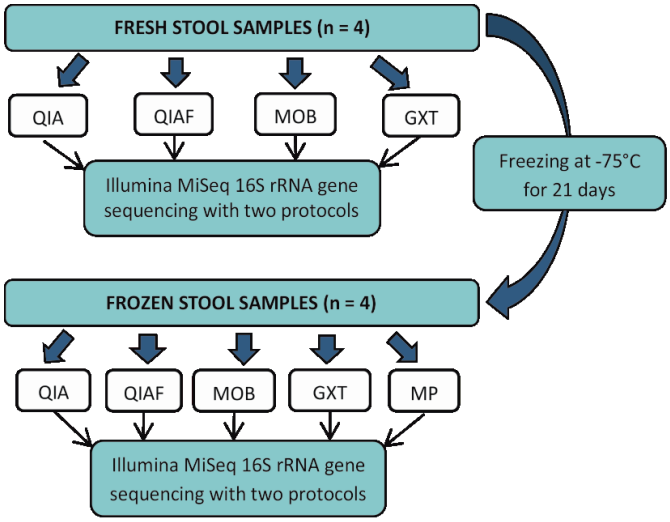

Figure 4. Workflow of the methodological study (original publication I + unpublished results). Abbreviations: QIA = QIAamp DNA Stool Mini Kit (QIAGEN GmbH); QIAF = QIAamp Fast DNA Stool Mini Kit (QIAGEN GmbH); MOB = PowerFecal DNA Isolation Kit (MO BIO Laboratories, Inc.), GXT = GXT Stool Extraction Kit VER 2.0 (Hain Lifescience GmbH); MP = MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche Diagnostics GmbH).

4.1.2 16S rRNA gene sequencing

Bacterial 16S rRNA gene profiles of the stool specimens were analyzed by two distinct Illumina MiSeq 16S rRNA gene sequencing protocols, one detecting the V3-V4 hypervariable regions and the other detecting the V4-V5 hypervariable regions of the bacterial 16S rRNA gene.

4.1.2.1 V3-V4 library preparation

The hypervariable regions V3 and V4 of the bacterial 16S rRNA gene were amplified by strictly following the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Illumina, 2014). Briefly, the V3-V4 region of the 16S rRNA gene was first amplified using gene-specific primers (Klindworth et al., 2013) that included specific Illumina overhang adapter nucleotide sequences

(Table 3; Figure 5). Then, sample-specific multiplexing indices and Illumina sequencing adapters were added to the gene-specific sequences in a multiplexing PCR step (Illumina, 2014). The expected PCR product size was approximately 550 bp.

Table 3. Sequences of the PCR primers used for encoding the 16S rRNA gene

16S rRNA gene region		Primer sequence (5' - 3')
V3-V4	forward primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG
	reverse primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC
V4-V5	forward primer	AATGATACGGCGACCACCGAGATCTACAC-i5*-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
	reverse primer	CAAGCAGAAGACGGCATACGAGAT-i7*-AGTCAGTCAGGCCCCGTCAATTCMTTTRAGT

*i5 and i7 represent the 8-nucleotide index sequences

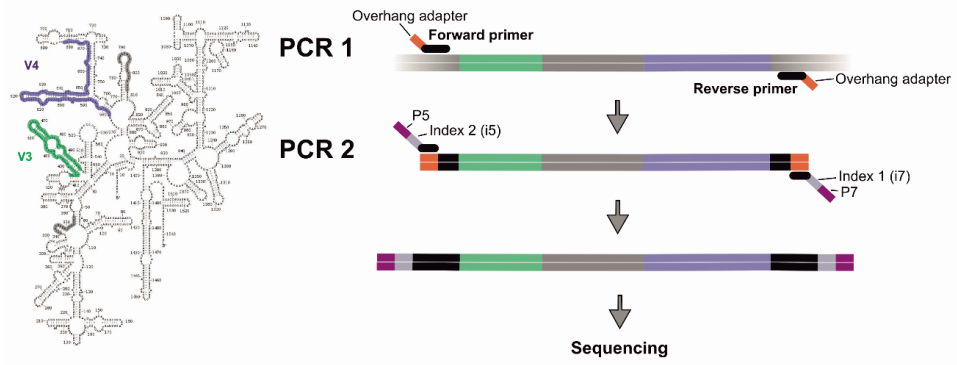

Figure 5. Library preparation protocol for V3-V4 sequencing. First, the 16S rRNA gene was amplified with gene-specific primers including specific Illumina overhang adapters. Then, in a second PCR, sample-specific indices and Illumina sequencing adapters (P5 and P7) were added to the gene-specific sequences. The 16S rRNA secondary structure of *E. coli* has been adapted from XRNA Gallery (http://rna.ucsc.edu/rnacenter/xrna/xrna_gallery.html), and the primer binding sites are highlighted in grey. The hypervariable regions are highlighted based on (Chakravorty et al., 2007).

4.1.2.2 V4-V5 library preparation

Hypervariable regions V4 and V5 of the bacterial 16S rRNA gene were amplified in a single PCR (Figure 6) using KAPA HiFi PCR kit (KAPA Biosystems, Wilmington, MA, USA) and applying custom-designed indexed primers (Table 4) modified from (Kozich et al., 2013). Each PCR reaction included 0.3 µM of the primers, 0.3 mM of dNTPs, 0.5 U of polymerase enzyme, and 50 ng of DNA template. The PCR program with Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) consisted of an initial denaturation at 98°C for 4 min, 25 cycles at 98°C for 20 s, 65°C for 20 s and 72°C for 35 s, and a final elongation at 72°C for 10 min. The expected PCR product size was approximately 500 bp.

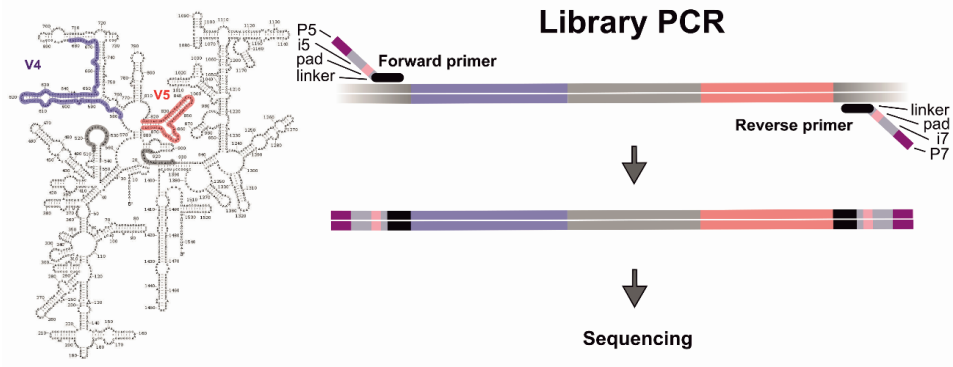

Figure 6. Library preparation protocol for V4-V5 sequencing. The 16S rRNA gene was amplified with gene-specific primers including Illumina sequencing adapters (P5 and P7), sample specific indices (i5 and i7), and specific pad and linker sequences (Kozich et al., 2013). The 16S rRNA secondary structure of *E. coli* has been adapted from XRNA Gallery (http://rna.ucsc.edu/rnacenter/xrna/xrna_gallery.html), and the primer binding sites are highlighted in grey. The hypervariable regions are highlighted based on (Chakravorty et al., 2007).

4.1.2.3 Illumina MiSeq 16S rRNA gene sequencing

The PCR products were purified with Agencourt AMPure XP Magnetic beads (Beckman Coulter, Inc., Brea, CA, USA) on a DynaMag™-96 magnenic plate (Life Technologies), and the PCR products were quality controlled with TapeStation (Agilent Technologies, Inc., Santa Clara, CA, USA). The final DNA concentrations of the purified 16S rRNA gene libraries were measured with Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA), and the libraries were then mixed in equal molar concentrations to generate a library pool with 4

nM concentration. The pool was denatured (Illumina, 2014), diluted into 4 pM concentration, and spiked with 25% denatured PhiX control (Illumina, Inc., San Diego, CA, USA). Paired-end sequencing (2 × 300 bp) was performed with Illumina MiSeq system, using MiSeq v3 reagent kit (Illumina, Inc.).

4.1.3 16S rRNA gene sequence data analysis

The initial raw sequence quality check was performed with FastQC quality-control program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and data were processed with Qiime (v. 1.9) pipeline (Caporaso et al., 2010, Kuczynski et al., 2012, Bokulich et al., 2013). The sequence reads were filtered with a quality score acceptance threshold 20, and chimeric sequences were filtered using usearch (v. 6.1). Operational taxonomic units (OTUs) were picked using uclust algorithm with 97% sequence similarity, excluding OTUs representing less than 0.05% of the total sequences. Then, to minimize inter-sample variation in sequencing efficiency, the OTUs of each sample were rarefied to a common sequencing depth by random sampling without replacement (Weiss et al., 2017). GreenGenes database (DeSantis et al., 2006) was applied for the OTU annotations.

All analyses of the 16S rRNA gene sequencing data were made from the rarefied OTU tables. Taxonomic summary produced by Qiime was visualized as bar charts, and statistical differences in the taxonomic richness, i.e. in the OTU abundances on taxonomic levels phylum and genus, were assessed with non-parametric Kruskal-Wallis test. False Discovery Rate (FDR) adjusted p-values < 0.05 were considered as statistically significant. OTUs existing in less than 25% of the samples were excluded prior to statistical testing.

Statistically significant differences in α-diversity were assessed with Kruskal-Wallis test from diversity metrics calculated by Qiime. P-values < 0.05 were considered as statistically significant. In this thesis, an additional cut-point of $p < 0.01$ has been used to emphasize the most distinct findings. Obvious outliers were excluded before the analyses. The differences in the overall bacterial diversity across the samples were inspected from Principal Coordinate Analysis (PCoA) plots produced by Qiime. The PCoA plots were visualized with EMPeror (Vázquez-Baeza et al., 2013). To confirm the visual observations, adonis analyses (Navas-Molina et al., 2013) were performed.

4.2 Experimental design of the mouse studies (II & III)

Two mouse experiments were performed to study the effects of gut microbiota modification on the emergence of metabolic disorders. The basic study design of the experiments was similar (Figure 7). In both experiments, two control groups, one on high-fat diet (HFD) and the other on chow diet, were given sterile phosphate buffered saline (PBS) intragastrically twice a week, every other week. At the same time, a bacterial treatment group on HFD was intragastrically administered with approximately 2×10^8 live bacterial cells dissolved in PBS twice a week, every other week. Both experiments lasted for 13 weeks, after which the mice were sacrificed and their whole blood, gut content, gut tissue, adipose tissue, and liver were collected. The animal experiments were approved by the National Animal Experiment Board in Finland (license: ESAVI/7258/04.10.07/2014), and all animal work was performed in accordance with the guidelines of the Act on the Use of Animals for Experimental Purposes by the Ministry of Agriculture and Forestry, Finland.

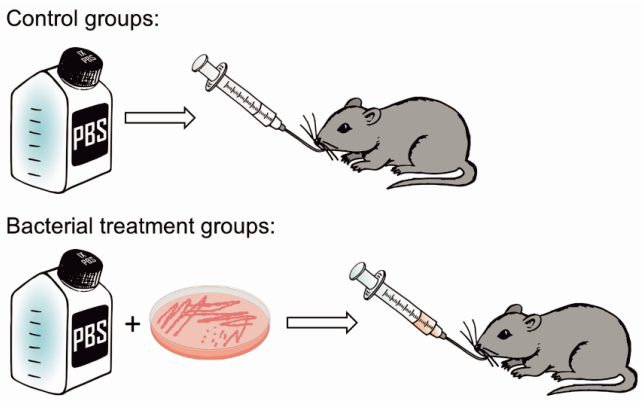

Figure 7. Simplified schematic presentation of the mouse studies (original publications II and III). Two control groups, one receiving HFD and the other receiving chow diet, were intragastrically given PBS twice a week, every other week. The bacterial treatment group received approximately 2×10^8 live bacterial cells, either *F. prausnitzii* or *E. cloacae*, twice a week, every other week. The experiments lasted for 13 weeks, after which the mice were sacrificed and their tissues were collected.

4.2.1 Mice

7-week-old C57BL/6N female mice were purchased from Charles River laboratories (Germany) via Scanbur. At the age of 8 weeks, the mice were randomly divided into HFD control, chow control and bacterial treatment groups (n = 6 per group). Baseline stool samples were collected into Nunc® CryoTubes (Sigma-Aldrich, Saint Louis, MI, USA), immersed into liquid nitrogen, and stored at -80°C. During the 13-week experiments, the mice were housed in individually ventilated cages under SPF conditions, three mice per cage. The mice received food and water *ad libitum* and were maintained on a 12/12-hour light/dark cycle. Upon arrival, all mice received irradiated chow diet (58124 DIO Rodent Purified Diet w/10% energy from fat) purchased from Labdiet/Testdiet, UK. When the experiment started, the HFD control group and the bacterial treatment groups were switched to chow-matching HFD (58126 DIO Rodent Purified Diet w/60% energy from fat), while the chow control group continued on the chow diet throughout the experiment (Table 4). Food intake in each cage was monitored at four or five different time points during the experiment, by weighing the consumed food per cage in 24-hour periods. Food intake per mice was then estimated by dividing the food consumption of the cage by the number of mice living in the cage. Body weight of the mice was measured weekly, always at the same time of the day. One mouse from the HFD control group and one from the *E. cloacae* treatment group were excluded from the study, as they suffered from significant weight loss during the treatment period.

Table 4. Composition of the diets

	Chow diet[a]		**High-fat diet[b]**	
Ingredient	**%**	**% kcal**	**%**	**% kcal**
Protein	16.9	18.0	23.1	18.1
Carbohydrates	67.4	71.8	25.9	20.3
Fat	4.3	10.2	34.9	61.6
Fiber	4.7		6.5	
kcal/g (kJ/g)	3.76 (15.7)		5.10 (21.3)	

[a]TestDiet® DIO Rodent Purified Diet w/10 % Energy from Fat
[b]TestDiet® DIO Rodent Purified Diet w/60 % Energy from Fat

4.2.2 Bacterial cultures and intragastric bacterial administration

F. prausnitzii ATCC® 27766™ pure cultures were maintained at +37°C on yeast extract, casitone, fatty acid, and glucose (YCFAG) agar plates (modified from (Lopez-Siles et al., 2012) in Whitley A35 anaerobic workstation (Don Whitley Scientific, UK). Intragastric inoculums were prepared by suspending the cultured bacterial cells in sterile PBS at a turbidity-based estimated cell density of 9×10^8 CFU/ml. The volume of each portion, including approximately 2×10^8 bacterial cells, was 220 µl. The suspensions were prepared at anaerobic atmosphere using anaerobic PBS, and the dosing syringes were sealed with Parafilm® M to enable the viability of the bacteria prior to the administration. At the time of transferring the suspension into the dosing syringe, the viability of the suspended bacterial cells was confirmed by plating an aliquot of the suspension on YCFAG agar plate. The sealed syringes were then transported to the animal facility, and the bacterial suspensions were administered to the mice intragastrically.

E. cloacae subspecies *cloacae* ATCC® 13047™ pure cultures were maintained at +37°C on Fastidious anaerobe blood agar plates (Lab M Limited, Lancashire, UK). Intragastric inoculums were prepared similarly as the *F. prausnitzii* inoculums, by suspending the bacterial cells in sterile PBS at an approximate cell density of 9×10^8 CFU/ml. The volume of each inoculum was 220 µl, including approximately 2×10^8 bacterial cells. At the time of transferring the bacterial suspension into the dosing syringe, the viability of the bacteria was confirmed by plating an aliquot of the suspension on Fastidious anaerobe blood agar plate. The dosing syringes were sealed with Parafilm® M and transported to the animal facility, and the suspensions were administered to the mice intragastrically.

4.2.3 Tissue sample collection

After the 13-week treatment period, the overnight-fasted mice were euthanized by CO_2 exposure, and their whole blood was drawn by cardiac puncture. Euthanasia was confirmed by cervical dislocation. Whole blood samples, collected by the cardiac puncture, were transferred into serum tubes, and after adequate clotting of approximately 30 minutes, the samples were centrifuged at 10 000 × g for 5 minutes to separate serum from cells. The serums were then divided into several Eppendorf tubes and stored at -80°C.

All tissues were collected with sterile instruments. Separate instruments were used for each tissue type, and the instruments were disinfected in ethanol between each dissection. The subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were harvested and weighed with an electronic scale.

Approximately 3 mm pieces of the adipose tissues were fixed in 10x w/v Tris-buffered zinc fixative (2.8 mM calcium acetate, 22.8 mM zinc acetate, 36.7 mM Zinc chloride in 0.1 M Tris-buffer, pH 7.4) for 24 hours at RT. The fixed tissues were washed with H_2O and PBS, 1 hour at RT with each, and then stored in PBS at +4°C until embedded in paraffin. Rest of the adipose tissues were placed in Nunc® CryoTubes (Sigma-Aldrich, Saint Louis, MI, USA), snap frozen by immerging in liquid nitrogen and stored at -80°C. These tissue samples were later pulverized in liquid nitrogen, using mortar and pestle, to obtain homogenous mixtures of the whole tissues for protein and gene expression analyses.

The liver tissue was harvested and weighed with electronic scale. Part of the right lobe of the liver was cut and embedded in Tissue-Tek® O.T.C. compound (Sakura® Finetek Europe, Leiden, Netherlands) and snap frozen in liquid nitrogen for immunohistochemical analyses. Rest of the liver was placed in Nunc® CryoTube, submerged in liquid nitrogen and stored at -80°C. For fat content analysis, along with protein and gene expression analyses, the tissue samples were later pulverized in liquid nitrogen. Left and right gastrocnemius muscle tissues were dissected, weighted with electronic scale and placed in Nunc® CryoTubes that were immediately submerged in liquid nitrogen and then stored at -80°C. For the protein and gene expression analyses, the tissue samples were later pulverized in liquid nitrogen. Last, the whole intestine was dissected, and the colon and cecum contents were collected into Nunc® CryoTubes, submerged immediately into liquid nitrogen and stored at -80°C. The intestinal tissue was weighed with electronic scale, transferred into Nunc® CryoTube, snap frozen in liquid nitrogen and stored at -80°C. Subsequently, the tissue samples were pulverized in liquid nitrogen for gene expression analyses.

4.2.4 Tissue sample analyses

4.2.4.1 Analyses of the serum samples

Serum glucose, glycerol, aspartate transaminase (AST), and alanine transaminase (ALT) were analyzed at the clinical laboratory of the Department of Sport and Health Sciences, University of Jyväskylä, using KONELAB 20XTi analyzer (Thermo Fischer Scientific, Vantaa, Finland) and standard KONELAB reagents (Thermo Fischer Scientific, Vantaa, Finland).

4.2.4.2 Liver triacylglycerol measurements and AST and ALT analyses

Total lipids from the liver tissues were extracted by a method modified from (Folch et al., 1957). Approximately 30 mg of the pulverized tissue was homogenized in 2:1 (v/v) chloroform/methanol ($CHCl_3$:MeOH) and washed with 0.9 % KCl. Phases were separated by centrifugation at 2000 × g for 3 min. The lower organic phase was saved, and for the upper phase, 86:14 (v/v) $CHCl_3$:MeOH was added and the extraction was repeated. Then the upper phase was discarded, and both organic phases were combined and evaporated to dryness under nitrogen stream. The dried lipid extract was dissolved in ethanol, and the TAG content was determined with KONELAB 20XTi analyzer. In study II, a more comprehensive additional fraction isolation method was used in order to enable fatty acid methyl ester analyses by gas chromatography (method described in original publication II).

To analyze AST and ALT levels directly from liver, approximately 20 mg of pulverized liver tissue was transferred into ice-cold lysis buffer (10 mM Tris-HCl, 150 mM NaCl2, 2 mM EDTA, 1% Triton X-100, 10% glycerol and 1 mM DTT) supplemented with protease and phosphatase inhibitors (Sigma Aldrich, St Louis, USA) and homogenized with TissueLyser (Qiagen, Valencia, CA, USA). After centrifugation at 12 000 x g for 10 min, AST and ALT were measured from the soluble liver protein extract with KONELAB 20XTi analyzer (Thermo Fischer Scientific, Waltham, MA, USA).

4.2.4.3 Gene expression analyses

Total RNA was extracted from the pulverized tissues using TissueLyser (Qiagen, Hilden, Germany) and Trizol reagent (Invitrogen, Carlsbad, CA, USA), by following the manufacturer's protocol. The RNA concentrations were measured with NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the RNAs were then stored at -80°C. For gene expression analyses, RNA was reversely transcribed using High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA), by following the manufacturer's instructions. Real-time quantitative PCR (qPCR) analyses were performed according to the MIQE quidelines (Bustin et al., 2009), using either in-house designed primers (purchased from Invitrogen) or primers derived from Mouse Primer Depot (http://mouseprimerdepot.nci.nih.gov/), iQ SYBR Supermix, and CFX96™ Real-time PCR Detection System (Bio-Rad Laboratories, Richmond, CA, USA). The target genes and the sequences of all qPCR primers used in the mouse studies are presented in Table 5. The PCR

program consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and +72°C for 30 s, and finally 5 s at +65°C. The relative expression values were calculated against a standard curve with the CFX96™ Software and normalized to the expression level of either *ACTB* mRNA or PicoGreen-quantified dsDNA (Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen; Lundby et al., 2005). Each sample was analyzed in duplicate.

Table 5. Sequences of the qPCR primers used in this study

Target gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Original publication
Acc2	GAGGCTGCATTGAACACAAG	TGTTCTCGGCCTCTCTTCAC	II + III
Actb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	II + III
Adipoq	CCTGGAGAGAAGGGAGAGAAA	CGAATGGGTACATTGGGAAC	II + III
Adipor	GGAGTGTTCGTGGGCTTAGG	GCAGCTCCGGTGATATAGAGG	II + III
Ccl2	AGGTGTCCCAAAGAAGCTGTA	ATGTCTGGACCCATTCCTTCT	III
Cdkn1a	CGGTGTCAGAGTCTAGGGGA	AGGATTGGACATGGTGCCTG	II
Cs	GCCATGGCTCTACTCACTGC	ATTCGTGGAAGAAGCACTGG	II
Ddit4	GCCTCTGGGATCGTTTCTCG	GGTCAAGGCCCTCTTCTCTG	II
Dgat2	CGCAGCGAAAACAAGAATAA	GAAGATGTCTTGGAGGGCTG	II + III
Il1b	TGTGAAATGCCACCTTTTGA	GGTCAAAGGTTTGGAAGCAG	III
Insr	CAGCCGGATGGGCCAATGGGA	CTCGTCCGGCACGTACACAGAA	II + III
Mgll	AAAGTTTGTCGGAGAATCGG	AAAGTTTGTCGGAGAATCGG	III
Mmp9	AGACGACATAGACGGCATCC	CTGTCGGCTGTGGTTCAGT	III
Rela	TTTCGATTCCGCTATGTGTG	GAACGATAACCTTTGCAGGC	III
Tjp1	GAGCGGGCTACCTTACTGAAC	GAGCGGGCTACCTTACTGAAC	II + III
Tlr4	CAAGAACATAGATCTGAGCTTC AACCCGCT	GTCCAATAGGGAAGCTTTCTAGAG	III
Tlr5	AAGTTCCGGGGAATCTGTTT	GCATAGCCTGAGCCTGTTTC	II + III
Xbp1	TGCTGAGTCCGCAGCAGGTG	TGCTGAGTCCGCAGCAGGTG	III

4.2.4.4 Protein expression analyses

Proteins from the pulverized tissues were extracted at 4°C with TissueLyser (Qiagen, Hilden, Germany), using ice-cold lysis buffer (10 mM Tris-HCl, 150 mM NaCl2, 2 mM EDTA, 1% Triton X-100, 10% glycerol and 1 mM DTT) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, Saint Louis, MI, USA). The protein concentrations were measured with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Approximately 20 to 40 µg of the protein extracts were separated by SDS-Page

using 4-20 % Criterion precast gradient gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred to nitrocellulose membranes. The membranes were visualized with Ponceau S staining and blocked with Odyssey Blocking Buffer (Li-COR Biosciences, Lincoln, NE, USA) for 1 hour at RT. The Western blot analyses were performed using primary antibodies against phospho-Acc (Ser79) phospho-Akt (Ser473), phospho-AS160 (Thr642), phospho-HSL (Ser660) (Cell Signaling Technology, Danvers, MA, USA), PGC1α (Abcam, Cambridge, UK), and Total OXPHOS Rodent WB Antibody Cocktail that includes antibodies against ATP5A, MTCO1, NDUFB8, SDHB, and UQCRC2 (Abcam, Cambridge, UK). Odyssey anti-rabbit IRDye 800 or anti-mouse IRDye 680 (LI-COR Biosciences, Lincoln, NE, USA) were used as secondary antibodies, and the blots were scanned and quantified with Odyssey CLX Infrared Imager (Li-COR Biosciences, Lincoln, NE, USA). If re-probing was necessary, the membranes were incubated for 10 min in 0.2 M NaOH at RT, washed with TBS and re-probed with appropriate antibodies. All samples and results were normalized to two Ponceau S stained bands due to differences in housekeeping GAPDH expression levels between the groups.

4.2.4.5 Histological and immunohistochemical analyses

For morphological analysis of the liver, 5 µm sections from the frozen liver tissues were cut with Leica CM3000 Cryostat (Leica, Wetzlar, Germany) at -24°C and stained with Hematoxylin & Eosin (H&E) using standard protocols. To evaluate the degree of liver steatosis (Levene et al., 2012), the sections were stained with Oil Red O as previously described (Weston et al., 2015). In addition, acetone-fixed liver sections were stained with Cy3-conjugated anti-smooth muscle actin (SMA; Sigma-Aldrich, Saint Louis, MO, USA). Imaging was done using an inverted wide-field microscope (Carl Zeiss, Oberkochen, Germany) with a confocal unit and 40× oil/1.4 NA objective (Carl Zeiss).

Paraffin-embedded adipose tissues were cut into 5 µm sections and stained immunohistochemically using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) to determine whether leukocytes had translocated into the adipose tissues. Staining was performed by following the manufacturer's instructions, using anti-mouse CD45 (clone 30F-11; BD Biosciences, Franklin Lakes, NJ, USA) as a primary antibody. Diaminobenzidine (Dako, Agilent Technologies, Santa Clara, CA, USA) was used as a chromogen, and counterstaining was done using Mayer's hematoxylin. The number of CD45 positive cells was calculated manually from the stained sections. On average, cells from approximately 10 fields of view were calculated, and the results were

presented as the number of cells per field. In addition, the adipocyte sizes from at least 500 randomly selected cells in each sample group were determined with CellProfiler 2.2.0.

4.2.4.6 DNA extraction from stool and colon samples

The DNA from approximately 100 mg frozen baseline stool samples and from post-treatment colon content samples were extracted with GTX Stool Extraction Kit VER 2.0 and GenoXtract® instrument (Hain Lifescience, Nehren, Germany) combined with an additional bead-beating in 0.1 mm Glass Bead Tubes (MO BIO Laboratories, Inc.) with MO BIO PowerLyzer™ 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc.) at 1000 rpm for 3 minutes. DNA concentrations of the extracts were measured with Qubit dsDNA HS assay kit and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). The DNAs were stored at -75°C until the 16S rRNA gene library preparation and sequencing (described in chapter 4.1.2.2).

4.2.5 Statistical analyses

Statistical analyses of the mouse studies were performed with either IBM SPSS Statistics 22 (IBM, Armonk, NY, USA) or JMP Pro 12 (SAS Institute, Inc., Cary, NC, USA). All data were checked for normality using Shapiro-Wilk´s test. For body weight, one-way ANOVA and Tukey's range test were used to analyze the differences between the study groups. The group differences in gene expression and protein phosphorylation levels, liver TAG content, and in the numbers of leukocytes were analyzed with non-parametric Kruskal-Wallis test and Mann Whitney U test, as the data were not normally distributed. *P*-values < 0.05 were considered as statistically significant. In this thesis, an additional cut-point $p < 0.01$ has been used to emphasize the most distinct findings.

5 RESULTS

5.1 Effects of sample storage, DNA extraction, and sequencing protocol on the 16S rRNA gene sequencing results (I)

All evaluated commercial DNA extraction kits performed sufficiently in extracting DNA from human stool specimens for NGS analysis. The DNA gain (µg per gram of feces) was, however, significantly higher with GXT than with any other method in both fresh and frozen specimens (Steel-Dwass All Pairs $p < 0.01$ for all). In addition, for frozen specimens, extraction with QIA resulted in a significantly higher DNA gain than the extractions with MOB or QIAF ($p < 0.01$ for both).

5.1.1 DNA extraction method had relatively little impact on the 16S rRNA gene sequencing results

The observed bacterial diversity of the fecal samples, represented as average Shannon indices, was not dependent on the DNA extraction protocol ($p = 0.91$ for fresh samples [unpublished data] and $p = 0.89$ for frozen samples). Inter-individual differences, by contrast, were explicit (Kruskal-Wallis $p < 0.01$). In principal coordinate analysis (PCoA) plot, as well as in Jack-Knifed Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Tree, the subsamples of each original specimen clustered together, irrespective of the DNA extraction method (Original publication I, Figures 4 and 5). Furthermore, adonis analysis reported no differences between the DNA extraction methods ($p = 1.0$), whereas grouping by study subjects was statistically significant ($p < 0.01$).

When only the frozen samples were assessed (original publication I), the phylum level bacterial composition of the samples, i.e. the OTU abundances, was not dependent on the DNA extraction method. In V4-V5 sequencing, samples extracted with MP tended to have slightly higher *Firmicutes*-to-*Bacteroidetes* ratios compared to the samples extracted with other methods ($p = 0.08$), but this tendency was not observed in the V3-V4 sequencing ($p = 0.2$). No other phylum level differences were observed between the DNA extraction methods in neither V3-V4 nor V4-V5 sequencing. In the bacterial genus level, on the other hand, the abundances of genera *Coprococcus* and *Dorea* appeared to significantly vary between samples extracted with distinct protocols (FDR < 0.05 for both). This was observed in both V3-V4 and V4-V5 sequencing. However, the overall abundance of both these genera was extremely low.

When fresh and frozen samples were analyzed together (unpublished data), Qiime reported statistically significant differences in V4-V5 sequencing for phyla *Actinobacteria* and *Proteobacteria* (FDR < 0.05 for both); the abundance of *Actinobacteria* was highest in the samples extracted with GXT or MP, while the abundance of *Proteobacteria* was highest in the samples extracted with QIAF. With V3-V4 sequencing, no differences in the phylum level bacterial composition were observed between the extraction protocols. Further, no DNA extraction -dependent differences in the *Firmicutes*-to-*Bacteroidetes* ratio were observed with neither V3-V4 nor V4-V5 sequencing when the fresh and frozen samples were analyzed together. By contrast, several genus level differences were observed between the DNA extraction methods: along with *Coprococcus* and *Dorea*, the abundance of *Blautia*, *Ruminococcus*, *Dialister*, and *Odoribacter* varied significantly with both V3-V4 and V4-V5 sequencing (FDR < 0.05 for all). Further, in V4-V5 sequencing, the abundance of genus *Faecalibacterium* differed between the extraction protocols (FDR < 0.05), while in V3-V4 sequencing, the abundance of genus *Oscillospira* varied significantly between the extraction protocols (FDR < 0.05).

5.1.2 Freezer storage tended to affect the Firmicutes-to-Bacteroidetes ratio of the fecal samples

The 21-day freezer storage had a divergent effect on the DNA gain (μg per g of feces) between the different extraction methods (unpublished data). With QIA, the DNA gain was significantly higher after the freezer storage (Mann-Whitney U test $p = 0.04$), whereas with MOB and QIAF, the DNA gain was significantly lower after the freezer storage ($p < 0.01$ for both). With GXT, no significant differences in the DNA gain were observed between the fresh and frozen specimens ($p = 0.25$). MP extractions were performed only for the frozen specimens, and thus no comparisons could be made. The MP results were, however, included in the analyses comparing the fresh and frozen specimens, because based on the results of original publication I, this was expected to have little impact on the results.

The observed α-diversity of the samples, represented as average Shannon indices, was not affected by the freezer storage ($p = 0.42$; unpublished data). Nevertheless, especially in V4-V5 sequencing, the freezer storage tended to affect the bacterial profiles produced by the 16S rRNA gene sequencing: In V4-V5 sequencing, the abundance of phylum *Firmicutes* was significantly higher in the frozen specimens (FDR < 0.05), whereas *Bacteroidetes* abundance tended to be higher in the fresh specimens (FDR = 0.054). Accordingly, the *Firmicutes*-to-

Bacteroidetes ratio tended to be lower in the freshly isolated specimens (p = 0.06). Further, genus level differences between the fresh and frozen fecal samples were seen in the abundances of *Dorea*, *Bifidobacterium* and *Faecalibacterium*; the abundances of all these genera were significantly higher in the frozen specimens (FDR < 0.05 for all; unpublished data). In V3-V4 sequencing, on the other hand, no statistically significant differences were observed in the abundance of any bacterial phyla or genera between the fresh and frozen specimens, but again, the *Firmicutes*-to-*Bacteroidetes* ratio tended to be lower in the freshly extracted samples (p = 0.09). Further, when the V3-V4 and V4-V5 sequencing results were analyzed together, the shift in the *Firmicutes*-to-*Bacteroidetes* ratio appeared statistically significant (p < 0.05). However, when the samples extracted with MP were excluded from the analysis, the difference became statistically insignificant (p = 0.16).

5.1.3 16S rRNA gene sequencing results were highly dependent on the 16S rRNA gene library preparation protocol

The sequencing results produced with the two distinct 16S rRNA gene library preparation protocols differed significantly from each other (original publication I). The average OTU counts of the samples were significantly higher with V4-V5 sequencing (Mann-Whitney U test p < 0.01), whereas the average Shannon indices were significantly higher with V3-V4 sequencing (p < 0.01). The samples analyzed with the different protocols clustered separately in the PCoA plot (Original publication I, Figure 7), and Jack-Knifed UPGMA tree revealed two distinct clusters. Adonis analysis affirmed that a remarkable portion of variation between the samples could be attributed to the 16S rRNA gene sequencing protocol (p < 0.01).

When observing the taxonomic bacterial composition of the samples, the abundances of phyla *Actinobacteria, Bacteroidetes, Proteobacteria*, and *Firmicutes* differed significantly between the V3-V4 and V4-V5 sequencing (FDR < 0.05 for all); the average *Bacteroidetes* abundance was higher with V4-V5 sequencing, whereas the abundances of the other phyla were higher with V3-V4 sequencing. In addition, the *Firmicutes*-to-*Bacteroidetes* ratio was significantly higher in V3-V4 sequencing (p < 0.01). Further, in the bacterial genus level, several statistically significant differences were observed between the V3-V4 and the V4-V5 sequencing protocols (original publication I). The results concerning the taxonomic bacterial profiles remained basically unaltered when the freshly-isolated and frozen samples were analyzed together (unpublished data).

5.2 Effects of intragastric *F. prausnitzii* administration on mouse metabolism and liver fat accumulation (II)

No statistically significant differences in long-term food intake were observed between the study groups, even though the food consumption of the HFD controls seemed to be slightly elevated compared to the chow controls and the *F. prausnitzii* -treated mice. At the endpoint of the study, no statistically significant differences in serum glycerol or glucose concentrations were observed between the study groups.

5.2.1 F. prausnitzii treatment reduced hepatic fat accumulation

The mice treated with *F. prausnitzii* had reduced liver TAG levels compared to the HFD controls ($p < 0.05$; Table 6), and their hepatic fat content, visualized by Oil Red O staining, was significantly lower. In addition, less lipid ballooning and liver fibrosis were observed in the *F. prausnitzii* -treated mice and the chow controls compared to the HFD controls (Original publication II, Figure 1). Based on gas chromatography analysis, the molar percentages of hepatic lipid classes 18:0 (stearate), 20:4n-6 (arachidonic acid), 20:5n-3 (eicosapentaenoic acid), and 22:6n-3 (docosahexanoic acid) in TAGs were significantly reduced in the *F. prausnitzii* -treated mice compared to the HFD controls. In chow controls, majority of the lipid classes in TAGs were reduced compared to the HFD controls. Furthermore, the percentages of several fatty acids in phospholipids were decreased in both the *F. prausnitzii* -treated mice and the chow controls compared to the HFD controls. The *F. prausnitzii* -treated mice and the chow controls expressed more TAG-synthesizing diacylglycerol-acyltransferase (*Dgat2*) and fatty-acid synthesizing acetyl coenzyme carboxylase (*Acc2*) mRNA than the HFD controls ($p < 0.05$ for all), but compared to the HFD controls, the *F. prausnitzii* -treated mice also expressed more lipid metabolism -enhancing adiponectin receptor (*Adipor*; $p < 0.01$). Chow controls did not increase *Adipor* expression but instead significantly increased the insulin receptor (*Insr*) expression ($p < 0.01$). Furthermore, the *F. prausnitzii* -treated mice had significantly lower hepatic AST and ALT values compared to the HFD controls ($p < 0.05$ for both) and more Ser^{79}-phosphorylated Acc ($p < 0.05$).

Table 6. Effects of the *F. prausnitzii* treatment on the physiology and tissue-specific gene/protein expression of the HFD-fed C57BL/6N mice

Tissue	Effects of the *F. prausnitzii* treatment*
Liver	↓ Triacylglycerol (TAG) levels ↓ Lipid ballooning ↑ Diacylglycerol-acyltransferase (*Dgat2*) expression ↑ Acetyl coenzyme carboxylase (*Acc2*) expression ↑ Adiponectin receptor (*Adipor*) expression ↑ Acc phosphorylation ↓ Hepatic AST and ALT levels
Subcutaneous adipose tissue (SAT)	↑ SAT mass ↓ Number of CD45+ cells ↑ AS160 phosphorylation
Visceral adipose tissue (VAT)	↓ Number of CD45+ cells ↑ VAT-specific adiponectin (*Adipoq*) expression ↑ Insulin receptor (*Insr*) expression ↑ Hormone sensitive lipase (HSL) phosphorylation
Muscle	↑ Gastrocnemius muscle mass ↑ ATP5A, UQCRC2, and MTCO1 expression
Gut	↑ Tight junction protein-encoding gene (*Tjp1*) expression ↑ *Firmicutes* abundance ↓ *Bacteroidetes* abundance

*Statistically significant differences between the *F. prausnitzii* -treated mice and the HFD controls are presented. ↑ = increase; ↓ = decrease.

5.2.2 F. prausnitzii treatment reduced adipose tissue inflammation and appeared to increase insulin sensitivity in VAT

The *F. prausnitzii* -treated mice had significantly higher SAT mass than the chow controls ($p < 0.01$) and the HFD controls ($p < 0.05$; Table 6). The number of CD45-positive cells in the SAT of the *F. prausnitzii* -treated mice was, however, significantly lower than in the HFD controls ($p < 0.05$), and the phosphorylation of AS160 was increased in comparison with the control groups ($p < 0.05$ for both).

The differences in VAT mass were not significant between the *F. prausnitzii* -treated mice and the HFD controls (Original publication II, Figure 3), but the number of CD45-positive cells in the VAT of *F. prausnitzii* treatment group was significantly lower ($p < 0.01$). Further, in comparison to the HFD control mice, the adipocytes of the *F. prausnitzii* -treated mice appeared smaller, and they expressed more visceral fat specific adipokine, adiponectin (*Adipoq*; $p < 0.05$). In addition, the VAT of the *F. prausnitzii* -treated mice expressed more *Insr* ($p < 0.05$) than the HFD controls, while the insulin-responsive hormone sensitive lipase (HSL)-ser^{660} phosphorylation was elevated ($p < 0.05$). The *F. prausnitzii*

treatment did not, however, affect the expression of cytokine interleukin-1 beta, *Il1b* (unpublished data).

5.2.3 *F. prausnitzii* treatment increased gastrocnemius muscle size and modified the gut microbiota composition

F. prausnitzii -treated mice had significantly higher gastrocnemius muscle mass than the control mice (compared to the HFD controls, $p < 0.05$ for the left gastrocnemius and $p < 0.01$ for the right gastrocnemius; Table 6). In addition, the *F. prausnitzii* -treated mice expressed more mitochondrial ATP5A, UQCRC2, and MTCO1 ($p \leq 0.05$ for all) compared to the HFD controls. No differences in the expression levels of peroxisome proliferator -activated receptor coactivator 1α (PGC1α), NDUFB8, and SDHB, or in the phosphorylation levels of Acc, AS160, or HSL, were observed (data not shown).

While the pre-treatment mouse fecal samples had similar phylum level bacterial profiles, the post-treatment samples differed between the study groups. Most prominently, the *F. prausnitzii* treatment seemed to increase the *Firmicutes*-to-*Bacteroidetes* ratio of the gut microbiota (Original publication II, Figure 6A); the *F. prausnitzii* -treated mice had significantly less *Bacteroidetes* and more *Firmicutes* than the HFD control mice ($p < 0.05$ for both), and a similar trend was also seen between the *F. prausnitzii* treatment group and the chow controls ($p = 0.07$ for both). At the genus level, the *F. prausnitzii* -treated mice had, for example, more *Lactobacillus* and *Streptococcus* bacteria than the control groups ($p < 0.05$ for both). In addition, compared to the HFD controls, the *F. prausnitzii* -treated mice expressed more intestinal tight junction protein-encoding *Tjp1* mRNA ($p < 0.05$).

5.3 Effects of intragastric *E. cloacae* administration on mouse metabolism and liver fat accumulation (III)

No differences were observed in the weight gain of the HFD-fed study groups during the *E. cloacae* experiment (Original publication III, Figure 1A). By contrast, in the second and third week of the study, the *E. cloacae* -treated mice weighted significantly more than the chow controls ($p < 0.05$ for both), while at the endpoint of the experiment, both the *E. cloacae* -treated mice and the HFD controls weighted significantly more than the chow controls ($p < 0.01$ and $p < 0.05$, respectively).

5.3.1 E. cloacae treatment increased serum AST values but not hepatic steatosis

The *E. cloacae* -treated mice had significantly higher serum AST values compared to the chow controls ($p < 0.01$) and the HFD controls ($p < 0.05$; Table 7). TAG-synthesizing *Dgat2* expression was increased in the *E. cloacae* -treated mice compared to the HFD controls ($p < 0.05$), but the liver TAG content did not significantly differ between the study groups. The expression of fat metabolism -enhancing *Adipor* was, however, increased in the *E. cloacae* -treated mice compared to the control groups ($p < 0.05$ for both). On the other hand, *E. cloacae* -treated mice had decreased AS160 phosphorylation levels compared to the control mice ($p < 0.05$ for both; Original publication III, Figure 5). The H&E staining revealed significantly less lipid ballooning in the chow controls compared to the HFD controls and the *E. cloacae* -treated mice, and in SMA staining, *E. cloacae* -treatment group seemed to express more SMA than the control groups (Original publication III, Figure 6).

Table 7. Effects of *E. cloacae* treatment on the physiology and tissue-specific gene/protein expression of the HFD-fed C57BL/6N mice

Tissue	Effects of the *E. cloacae* treatment*
Serum	↑ Serum AST ↑ Serum glycerol
Liver	↑ Diacylglycerol-acyltransferase (*Dgat2*) expression ↑ Adiponectin receptor (*Adipor*) expression ↓ AS160 phosphorylation
Subcutaneous adipose tissue (SAT)	↑ Fat mass ↓ Insulin receptor (*Insr*) expression ↑ *Mgll* expression ↑ Proportion of hypertrophic adipocytes ↓ Number of CD45+ cells ↓ X-box binding protein 1 (*Xbp1*) expression
Visceral adipose tissue (VAT)	↑ VAT-specific adiponectin (*Adipoq*) expression ↑ Insulin receptor (*Insr*) expression ↑ NF-κβ p65 mRNA (*NFκB*) expression ↓ Akt phosphorylation

*Statistically significant differences between the *E. cloacae* -treated mice and the HFD controls are presented. ↑ = increase; ↓ = decrease.

5.3.2 *E. cloacae* treatment activated lipolysis, but also increased adiponectin expression in VAT

Compared to the chow controls, the *E. cloacae* -treated mice and the HFD controls had significantly increased VAT masses ($p < 0.05$ for both). The expression of hepatic fat oxidation -enhancing VAT-specific adipokine, *Adipoq*, was significantly higher in the *E. cloacae* -treated mice compared to the HFD controls ($p < 0.05$; Table 7). Compared to the HFD controls, the *E. cloacae* -treated mice and the chow controls had higher expression levels of NF-κβ p65 mRNA (*NFκB;* $p < 0.05$ and $p < 0.01$, respectively). In addition, the *E. cloacae* -treated mice and the chow controls expressed more *Insr* than the HFD controls ($p < 0.05$ and $p < 0.01$, respectively). On the other hand, the VAT of the *E. cloacae* -treated mice had lower phosphorylation levels of Akt-ser^{473} than the VAT of the control mice ($p < 0.05$ for both), and the phosphorylation of HSL-ser^{660} was decreased in comparison to the chow controls ($p < 0.05$). In addition, compared to the chow controls, the monoacylglycerol lipase (*Mgll)* expression in the VAT of the *E. cloacae* -treated mice was significantly elevated ($p < 0.01$).

5.3.3 *E. cloacae* treatment increased fat mass and the percentage of hypertrophic adipocytes in SAT

The SAT mass of *E. cloacae* -treated mice was elevated compared to the HFD and chow controls ($p < 0.05$ for both; Table 7). In addition, the expression level of *Insr* in the SAT of *E. cloacae* -treated mice was significantly lower than in the control groups ($p < 0.01$ for both). The SAT of the chow controls had significantly more phosphorylated Acc than the HFD controls ($p < 0.05$) and the *E. cloacae* treatment group ($p < 0.01$). In addition, the HSL-ser^{660} phosphorylation was higher in the chow controls than in the HFD controls ($p < 0.01$) and the *E. cloacae* -treated mice ($p < 0.05$). On the other hand, the *Mgll* expression was significantly higher in the *E. cloacae* -treated mice than in the HFD controls ($p < 0.05$) and chow controls ($p < 0.01$), and in accordance, the *E. cloacae* treatment group had significantly higher serum glycerol levels than the control mice ($p < 0.05$ for both). Based on H&E staining and cell size counting, the *E cloacae* treatment group had a higher proportion of hypertrophic (30-40 μM) adipocytes than the HFD control mice ($p < 0.01$). Nevertheless, the *E. cloacae* -treated mice had less endoplasmic reticulum (ER) stress -related spliced X-box binding protein 1 (*Xbp1*) than the HFD controls ($p < 0.05$) and significantly less CD45-positive cells in the SAT than the control groups ($p < 0.01$ for both).

6 DISCUSSION

6.1 16S rRNA gene sequencing results are highly dependent on the sample pre-processing protocols and the applied analysis methods

During the past decade, the methodological development in the NGS protocols has revolutionized the gut microbiota research. Ever since the high-throughput analysis protocols have become more cost-effective, they have also become more readily available, and consequently, the number of studies concerning the role of gut microbiota on human health has escalated (Tringe & Hugenholtz, 2008, Hamady & Knight, 2009). Along with large consortiums and population-wide study projects, e.g. The Human Microbiome Project (Turnbaugh et al., 2007), Metagenomics of the Human Intestinal Tract (Ehrlich & MetaHIT Consortium, 2011), and The American Gut Project (McDonald et al., 2015), a vast number of independent laboratories and study groups are performing high quality gut microbiota research. Consequently, the knowledge on the structure and functions of the gut microbes is continuously expanding. However, despite the constant development in the analysis protocols, standardization and comparability of the gut microbiota NGS studies remains a challenge (Cardona et al., 2012, Kennedy et al., 2014, Santiago et al., 2014, IHMS, 2015, Costea et al., 2017). Therefore, aiming at enhancing the data quality and result comparability, we analyzed the impact of different 16S rRNA gene targeting primers and different sample preprocessing methods, i.e. sample freezing and different DNA extraction methods, on the results of the NGS-based gut microbiota composition analysis. The results of this study reveal that the 16S rRNA gene sequencing protocol has a significant impact on the gut microbiota composition analysis results. Furthermore, our results show that the sample pre-processing protocols, i.e. freezer storage and DNA extraction, also affect the analysis results, but compared to the effect of the 16S rRNA gene library preparation protocol, the overall effects of the pre-processing appear relatively minor.

In this study, five different DNA extraction protocols, two storage conditions (fresh vs. frozen samples) and two 16S rRNA gene sequencing protocols (V3-V4 sequencing vs. V4-V5 sequencing) were evaluated in order to develop a well-working pipeline for gut microbiota composition analysis. As reported previously (Liu et al., 2008, Klindworth et al., 2013, Starke et al., 2014, Tremblay et al., 2015) and reaffirmed in this study, the selected 16S rRNA gene sequencing protocol has a major impact on the microbial community analysis results. In this study, both the observed bacterial diversities and the taxonomic profiles of the samples were highly dependent on the 16S rRNA gene sequencing protocol.

However, as both the 16S rRNA gene target region and the library preparation PCR protocol varied between the evaluated methods, it cannot be concluded whether the differences in the results occurred due to the gene target region or due to the differences in the PCR methodology. For example, in addition to the differences in the PCR protocols and chemistries, the ability of the applied primers to bind to the DNA of different bacterial species may have varied. Additionally, the V3-V4 sequencing and the V4-V5 sequencing were performed in different laboratories, which may have had a substantial effect on the results. Nevertheless, our findings underline the importance of careful evaluation of the 16S rRNA gene library preparation protocols. The storage conditions prior to the DNA extraction, on the other hand, mainly appeared to affect the *Firmicutes*-to-*Bacteroidetes* ratio of the samples. Even though the difference in the ratio was not statistically significant when samples extracted with MP were excluded from the analysis, our results suggest that freezer storage prior to the DNA extraction may slightly decrease the relative abundance of phylum *Bacteroidetes* in the fecal samples. The same phenomenon has been reported previously in a quantitative PCR based study (Bahl et al., 2012). The *Firmicutes*-to-*Bacteroidetes* ratio is a frequently used biomarker related to human physiology (Ley et al., 2006, Armougom & Raoult, 2008, Ley, 2010), but due to partially inconsistent results in some studies (Duncan et al., 2008, Collado et al., 2008, Schwiertz et al., 2010, Raman et al., 2013), the validity of the ratio remains controversial. Our findings suggest that the incompatibility between the studies may at least partly arise from differences in the sample pre-processing. At the same time, our V4-V5 sequencing results also indicate that the sample storage conditions may cause variation in the bacterial genus level composition. Of the observed differences, especially the higher relative abundance of *F. prausnitzii* in the frozen specimens may have importance, as *F. prausnitzii* is considered as a possible biomarker for a healthy human gut (Lopez-Siles et al., 2017, Martin et al., 2017). Thus, overall, the results of this study emphasize that standardization of the sample collection and storage is crucial in the gut microbiota composition studies, and that all samples analyzed together should be processed with identical methods. In addition, our results underline that comparisons of studies conducted with different analysis methods are not reliable.

Of the five DNA extraction methods evaluated in this study, QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) is one of the two DNA extraction methods recommended by the International Human Microbiome Standards (IHMS, 2015). It is a manual extractions protocol based on chemical cell lysis and DNA capture by silica membranes. QIAamp Fast DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) is a simplified version of QIAamp DNA Stool Mini Kit, yet still an entirely manual DNA extraction kit. This protocol also is based on chemical cell lysis and DNA capture by silica membranes.

PowerFecal DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), on the other hand, is a manual DNA extraction kit relying on bead-beating cell lysis and DNA capture by silica membranes. GXT Stool Extraction Kit VER 2.0 for GenoXtract® instrument (Hain LifeScience GmbH, Nehren, Germany) is a semi-automatic DNA extraction kit based on magnetic bead technology, while MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche Diagnostics GmbH, Mannheim, Germany) relies on chemical cell lysis and magnetic bead technology. Even though MagNA Pure is a high-throughput DNA extraction instrument, the extraction protocol includes several manual pre-processing steps and is thus considered to be semi-automatic in this study. To our knowledge, this was the first study to evaluate the usability of these semi-automatic DNA extraction methods in 16S rRNA gene sequencing -based gut microbiota composition analysis. Overall, there were no significant variation in the efficiency of the tested DNA isolation protocols; all kits performed sufficiently in extracting DNA from the fecal samples (see Chapter 5.1.1). However, when considering the amount of labor affiliated with each extraction protocol, the semi-automated methods clearly rose above the manual methods. This suggest that the semi-automatic methods offer a practical option to substitute the manual extraction kits in the sample pre-processing. This could significantly enhance the sample processing time and decrease the hands-on time in the NGS studies. In addition, even though not observed in this study, semi-automatic methods are likely to provide improved reproducibility compared to manual methods, as manual methods are more prone to human errors for example in pipetting. Use of automation could therefore possibly stabilize the quality of the DNA extractions and, to some extent, subsequently increase the comparability of 16S rRNA gene sequencing results between different laboratories.

The effect of DNA extraction on the results of microbial profile and diversity analyses has been extensively investigated in several previous studies (Salonen et al., 2010, Maukonen et al., 2012, Kennedy et al., 2014, Wesolowska-Andersen et al., 2014, Cruaud et al., 2014, Starke et al., 2014, Wagner Mackenzie et al., 2015, Costea et al., 2017), but due to partially conflicting results, no clear consensus on the matter has been reached. The inconsistences may have arisen from overall methodological differences, since in addition to evaluating different DNA extraction kits, variable sample collection and storage procedures have been used in the studies. Furthermore, the analysis methods have been variable. And on the other hand, it has been reported that even if two extraction protocols produce similar results for one sample, they may result in contradictory results for another (Cruaud et al., 2014), which suggests that the effects of the DNA extraction may be sample-dependent. Thus, the results of this study may not be fully comparable with any previous studies, as fecal samples from only four adult donors were analyzed. However, overall, the results of this study, along with previous studies

(Salonen et al., 2010, Wagner Mackenzie et al., 2015), indicate that inter-individual variation of the fecal samples clearly exceeds the variation resulting from the choice of the DNA extraction protocol. Altogether, our results suggest that all DNA extraction kits evaluated in this study are suitable to precede the 16S rRNA gene sequencing when studying inter-individual differences of the GI tract microbiota profiles. Yet, to enhance the validity of the results, the obtained methods should always be adequately optimized. Further, the results of this study are not applicable to other downstream protocols. For example, significant DNA extraction dependent differences have been reported in the results of shotgun metagenome sequencing (Costea et al., 2017).

6.2 *F. prausnitzii* has the potential to restrain liver fat accumulation, whereas *E. cloacae* may be able to cause liver damage

The underlying mechanisms in the pathogenesis of NAFLD and NASH are still incompletely understood. Currently, the most prevailing theory is that the induction and progression of hepatic steatosis and liver inflammation require multiple parallel factors to act together (reviewed in Buzzetti et al., 2016). These factors presumably include, for instance, genetic factors (Kantartzis et al., 2009), increased gut permeability (Miele et al., 2009), and adipose tissue dysfunction and inflammation (Tilg & Hotamisligil, 2006, Buzzetti et al., 2016). In addition, one important factor is IR, which in the liver promotes hepatic *de novo* lipogenesis and in the adipose tissue increases lipolysis that consequently increases the efflux of FFAs into the liver (Buzzetti et al., 2016, Bril et al., 2017). These factors seem to act both consecutively and simultaneously, and together predispose the liver to steatosis and lipotoxicity (Lomonaco et al., 2012, Bril et al., 2017). This lipotoxicity is suggested to induce oxidative stress and further impairment in the insulin signaling, as well as activation of several inflammatory cascades (Cusi, 2010, Lomonaco et al., 2012). The so-called multiple-hit hypothesis may explain why all obese individuals do not develop metabolic syndrome or NAFLD. In addition, it may explain why the progression of NAFLD varies highly between individuals, encompassing a wide spectrum of conditions from mild steatosis to severe NASH (Buzzetti et al., 2016).

Because gut microbes play a crucial role in regulating the intestinal permeability, bacterial-based treatment has been suggested to serve as a possible future method to treat or prevent NAFLD and other metabolic disorders (Cano et al., 2013, Lambert et al., 2015). In this study, we aimed at exploring the effects of intragastric bacterial treatment on host health and metabolism in C57BL/6N mice. The intragastric bacterial treatment was performed with either *F.*

prausnitzii (original publication II) or *E. cloacae* (original publication III). These intestinal bacterial species were selected, because several previous studies had reported *F. prausnitzii* to obtain health-promoting properties (Sokol et al., 2008, Miquel et al., 2014, Rossi et al., 2016), whereas *E. cloacae* had been associated with obesity and IR (Fei & Zhao, 2013). Further, a previous study by our research group had reported that human subjects with NAFLD possessed significantly decreased *F. prausnitzii* abundances and meanwhile tended to have slightly increased enterobacteria abundances in their fecal samples (Munukka et al., 2014). The effects of either *F. prausnitzii* or *E. cloacae* treatment were studied by comparing the bacterial-treated HFD-fed C57BL/6N mice to HFD control mice that served as models of NAFLD, and to chow control mice that served as healthy controls. Even though the chow controls acted as healthy controls, the comparison of the bacterial-treated mice and the HFD controls tended to be more informative in this study, because the effects of the bacterial treatments could not be comprehensively distinguished from the effects of the HFD.

The fat mass of the mice was elevated in both the *F. prausnitzii* as well as the *E. cloacae* treatment groups, which is a natural consequence of the HFD in C57BL/6 mice (Jo et al., 2009). As no bacterial treatment group fed with chow diet was included in the studies, it remains uncertain whether the fat mass of the mice would have also increased as a consequence of the intragastric bacterial administration e.g. due to excessive SCFA production (Bäckhed et al., 2004), or whether it was solely due to the HFD. Therefore, no firm conclusions about the role of the bacterial administration on the overall fat mass accumulation can be drawn in this study. However, the results of this study suggest that the effects of the *F. prausnitzii* treatment and the *E. cloacae* treatment on mouse metabolism were indisputably different. For example, the influence of the *F. prausnitzii* treatment on insulin signaling resembled the effects of the chow diet, suggesting that the *F. prausnitzii* treatment could avert the harmful effects of the HFD, whereas the results of the *E. cloacae* -treated mice rather indicated adipose tissue IR. In addition, based on the H&E staining and cell size counting, the *E. cloacae* treatment group had more hypertrophic (30-40 μM) adipocytes than the HFD controls. Hypertrophy, i.e. increased adipocyte size, together with hyperplasia, i.e. increased adipocyte number, are normal mechanisms for adipose tissue expansion (Rutkowski et al., 2015), but excess hypertrophy can lead to adipocyte hypoxia that is suggested to have an important role in the development of adipose tissue inflammation and IR (Olefsky & Glass, 2010, Sun et al., 2011). However, while the adipose tissue inflammation and IR are usually associated with ER stress and infiltration of leukocytes into the adipose tissue (Olefsky & Glass, 2010), both bacterial treatment groups had significantly less CD45-positive cells in their SAT in comparison to the control groups. In addition, the *E. cloacae* -

treated mice had less ER stress -related spliced *Xbp1* than the HFD controls. The expression of *Xbp1* was not studied in the *F. prausnitzii* experiment. The underlying reasons for these findings could not be identified.

6.2.1 Mechanisms by which F. prausnitzii treatment may enhance host health and metabolism

In this study, we observed that the *F. prausnitzii* (ATCC® 27766™) treatment decreased the hepatic fat content of the HFD fed mice; the hepatic fat content of the *F. prausnitzii* -treated mice was significantly low compared to the livers of the HFD controls. In addition, the *F. prausnitzii* -treated mice had significantly lower hepatic AST and ALT values than the HFD controls. In general, our findings regarding the hepatic lipid measurements, histological liver samples, and the AST and ALT values support each other and together suggest that the *F. prausnitzii* -treated mice had healthier livers than the HFD controls. Overall, our results thus indicate that *F. praus*nitzii had a capability to advert the negative effects of HFD and consequently restrain NAFLD in the HFD-fed mice.

Although the underlying mechanisms could not be fully elucidated in this study, certain effects of the bacterial treatment on the metabolic activities of the host were identified that could have contributed to the prevention of the liver fat accumulation. For instance, in the VAT, the expression of insulin receptor (*Insr*), as well as the insulin-stimulated HSL-ser^{660} phosphorylation, were significantly higher in the *F. prausnitzii* -treated mice and the chow controls in comparison to the HFD controls, which indicates that the VAT of the HFD controls may have been less insulin sensitive. Further, while obesity often is associated with adipose tissue inflammation and an infiltration of leukocytes, especially macrophages, into the adipose tissues, the number of CD45 positive cells in the adipose tissues of the *F. prausnitzii* -treated mice was decreased compared to the HFD controls, even though the fat mass was high in both groups. This suggests that the adipose tissues of the *F. prausnitzii* -treated mice may have been less inflamed. As previously mentioned, adipose tissue IR and inflammation not only affect the adipose tissue itself but play important roles in the overall metabolism. Adipose tissue IR, for instance, tends to increase TAG lipolysis, which subsequently leads to excess supply of the FFAs into the liver and thus predisposes the liver to excess hepatic fat accumulation (Lomonaco et al., 2012, Bril et al., 2017). Meanwhile, adipose tissue inflammation is considered as one of the key factors contributing to the pathogenesis of NAFLD, because in addition to further promoting the IR (Olefsky & Glass, 2010), it alters the secretion of adipokines and pro-inflammatory cytokines that can have systemic effects (Tilg &

Hotamisligil, 2006). As mesenteric VAT drains to hepatic portal system, liver may be especially exposed to cytokines secreted by this tissue (Rytka et al., 2011, Item & Konrad, 2012). Furthermore, significantly high adiponectin expression was observed in the VAT of the *F. prausnitzii* –treated mice in comparison to the HFD control mice. Adiponectin has been reported to obtain insulin-sensitizing effects on whole organism level, and low concentrations of this adipokine have been previously associated with obesity and related metabolic disorders (Liu et al., 2012, Caselli, 2014). Therefore, our results suggest that the *F. prausnitzii* treatment may have been able to block some of the adipose tissue related factors that, based on the multiple hit hypothesis (Buzzetti et al., 2016), play central roles in the development of NAFLD.

In addition to the suppression in adipose tissue IR and inflammation, the reduced liver fat accumulation of the *F. prausnitzii* -treated mice may be explained by enhanced hepatic fatty-acid oxidation and lipid clearance. Compared to the HFD controls, the *F. prausnitzii* -treated mice had high expression values of hepatic *Adipor*, which has previously been suggested to improve IR, enhance fat metabolism, and possess anti-inflammatory activities (Tilg & Hotamisligil, 2006). In addition, the *F. prausnitzii* -treated mice, as well as the chow controls, had significantly high hepatic Acc phosphorylation levels in comparison to the HFD controls, which possibly indicates reduced lipogenesis and enhanced fatty acid oxidation (Ha et al., 1994, Fullerton et al., 2013). Thus, the suppressed hepatic fat accumulation in the *F. prausnitzii* -treated mice in comparison to the HFD controls may have been due to enhanced hepatic fatty-acid metabolism. In general, the effects of the *F. prausnitzii* treatment on the liver of the mice largely resembled the effects of the chow diet. The chow controls, however, did not significantly increase the hepatic *Adipor* expression in comparison to the HFD controls, but instead significantly increased the hepatic insulin receptor (*Insr*) expression. This suggests that the liver metabolism of the chow controls differed from the *F. prausnitzii* -treated mice, which is not surprising, as the *F. prausnitzii* -treated mice had to process the excess fat in their diet. Retrospectively, it would have been extremely enlightening to also study a bacterial treatment group fed with chow diet.

One of the mechanisms through which *F. prausnitzii* possibly affected the health of the mice was its ability to favorably modulate the gut integrity by increasing *Tjp1* expression. This was quite expected, as *F. prausnitzii* is an important producer of butyrate, which has already previously been associated with increased gut epithelial integrity and alleviated intestinal inflammation (Chapman et al., 1994, Hamer et al., 2009). In fact, several studies have already reported that *F. prausnitzii* may be capable of improving the gut epithelial health (Benus et al., 2010, Carlsson et al., 2013, Martin et al., 2014). In addition, other

anti-inflammatory bacteria have been previously reported to support intestinal barrier function and the gut integrity (Eun et al., 2011, Bomhof et al., 2014), suggesting that certain bacterial species or bacterial fermentation products may be crucial for maintaining the intestinal health. However, based on the 16S rRNA gene sequencing, *F. prausnitzii* was not able to permanently inhabit the gut of the mice in this study. This was possibly either due to insufficient amount of inoculated bacterial cells or too few inoculations during the experiment. Alternatively, the GI tract of the mice may have not offered a favorable habitat for the bacterium, or the HFD may have prevented the long-term colonization. Yet even in these circumstances, the *F. prausnitzii* treatment was able to cause evident health effects and to modify the overall gut microbiota composition. This suggests that even a short-term colonization of the bacterium, possibly due to the anti-inflammatory compounds produced by it, is sufficient to induce beneficial effects on the host health. Interestingly, the *F. prausnitzii* treatment also increased the mouse muscle mass, which seemed to be linked to enhanced mitochondrial respiration. This finding supports the previous suggestion that gut microbes could affect the muscle size and metabolism (Bindels & Delzenne, 2013). Nevertheless, a bit surprisingly, we did not observe any differences in the expression of protein PGC1α, which is one of the main regulators of oxidative metabolism and has been previously related to muscle growth (Puigserver & Spiegelman, 2003, Bäckhed et al., 2007). Yet, as only the endpoint situation was studied, it is possible that the *F. prausnitzii* -treated mice had higher PGC1α expression levels at some earlier point during the experiment.

Taken together, the results of this study suggest that the intragastric *F. prausnitzii* treatment was able to advert the negative effects of HFD and protected the mice from NAFLD. Furthermore, our results suggest that the *F. prausnitzii* -treated mice were metabolically healthier than the HFD controls. Our findings are in line with several previous studies that have reported associations between *F. prausnitzii* supernatant and metabolic and immunological health of mice (Carlsson et al., 2013, Huang et al., 2016, Rossi et al., 2016, Wang et al., 2017). To our knowledge, this was the first study to show that intragastric *F. prausnitzii* administration could prevent liver fat accumulation in mice.

6.2.2 Possible mechanisms behind the harmful effects of E. cloacae treatment

The results of this study demonstrate that, contrary to the *F. prausnitzii* treatment, *E. cloacae subsp. cloacae* (ATCC® 113047™) administration induced adipose tissue hypertrophy and hepatic damage in the HFD-fed mice. Altogether, the *E. cloacae* treatment tended to have negative effects on the mouse

metabolism. For example, even though the *E. cloacae* treatment did not have a significant effect on the liver fat content in comparison to the HFD control mice, the treatment significantly elevated the serum AST values of the mice. Additionally, the *E. cloacae* treatment group seemed to express more SMA than the control groups, which along with the high serum AST values indicates liver damage. Thus, in conclusion, our results suggest that *E. cloacae* treatment was able to cause liver damage in the HFD-fed mice. Enterobacteria have already previously been associated with liver function; the abundance of phylum *Proteobacteria,* including the *Enterobacteriaceae* family, is suggested to be increased in NAFLD and NASH patients (Zhu et al., 2013, Shen et al., 2017) and increased enterobacteria abundances have also been reported in patients with liver cirrhosis (Chen et al., 2011). Furthermore, in accordance with our results, previous studies have reported that *E. cloacae* strain B29, a close relative to *E. cloacae* ATCC® 113047™, is able to induce obesity and liver injure in HFD fed GF mice (Fei & Zhao, 2013, Yan et al., 2016).

The mechanisms underlying the harmful effects of the *E. cloacae* treatment appeared unforeseeably complicated and thus remained largely unresolved in this study. For example, the reduced *Insr* expression in SAT, along with increased serum glycerol levels and increased *Mgll* expression in both SAT and VAT, indicate that the *E. cloacae* treatment may have caused partial insulin resistance that subsequently increased adipose tissue lipolysis, but this could not be confirmed in this study. Impaired regulation of lipolysis can cause adipocyte hypertrophy, which again is suggested to contribute to ER stress, adipose tissue inflammation, and further exacerbation of the adipose tissue IR (Olefsky & Glass, 2010, Sun et al., 2011, Makki et al., 2013, Morigny et al., 2016). However, as already mentioned, the *E. cloacae* treatment group had significantly less CD45-positive cells in their SAT in comparison to the control groups, and less ER stress -related spliced *Xbp1* than the HFD controls. Furthermore, since no insulin tolerance tests were performed, it cannot be confirmed that insulin resistance existed.

There were no differences in the insulin-stimulated HSL-ser^{660} phosphorylation, which inhibits the action of HSL, between the *E. cloacae* -treated mice and the HFD controls in neither SAT, VAT, or liver. Compared to the chow controls, on the other hand, both HFD fed groups had significantly low phosphorylation levels in each tissue. HSL is known to induce lipolysis, and it has been suggested to contribute to IR and NAFLD by releasing glycerol into the portal vein (Karpe et al., 2011). Meanwhile, the *E. cloacae* -treated mice, along with the chow controls, had significantly higher hepatic TAG-synthetizing *Dgat2* expression in comparison to the HFD controls, but the liver TAG content did not significantly differ between the study groups. This may at least partly arise from the increased

expression of the fat metabolism -enhancing *Adipor* in the *E. cloacae* -treated mice in comparison to the control mice, but the underlying reason for the elevated adipokine receptor expression remains unknown. In addition, the *Adipoq* expression in the VAT of the *E. cloacae* -treated mice was elevated – this finding is contrary to the previous results obtained with *E. cloacae* strain B29 (Fei & Zhao, 2013). On the other hand, compared to the control mice, the *E. cloacae* -treated mice had decreased AS160 phosphorylation levels, which potentially decreased the glucose incorporation into glycogen in liver (Hargett et al., 2016). It remains to be determined whether the contradictory results stem from strain-specific differences or some methodological reason such as inadequately frequent bacterial administrations. As the gut microbiota composition was not analyzed in this study, it is not definite that the colonization of the bacterium was successful, and furthermore, the effects of the *E. cloacae* treatment on the overall gut microbiota composition remain unknown. Further, it remains uncertain whether the *E. cloacae* administration alone would have been adequate to cause the effects observed in this study, or whether synergy with the HFD was required. In GF mice, *E. cloacae* and HFD have been suggested to possess a synergistic effect that contributes to obesity and metabolic disorders (Fei & Zhao, 2013, Yan et al., 2016). Thus, it is possible that also in conventionally bred mice *E. cloacae* is able to predispose the host to metabolic dysfunction and liver damage only when the intestinal barrier function is already hampered due to the HFD. This should be confirmed in future studies. Altogether, the results of this study indicate that the intragastric administration of certain bacterial species, in this case *E. cloacae*, is adequate to possess harmful effects on mouse metabolism, at least when combined with HFD.

6.3 *F. prausnitzii* as a possible future probiotic

The results of this study strengthen the supposition that *F. prausnitzii* has health-beneficial potential, and further suggest that the therapeutic use of this bacterium should be further studied. For example, humans having low abundance of *F. prausnitzii* in their GI tract could benefit from the targeted gut microbiota modulation. While previous studies have already reported that certain *Lactobacillus* strains are able to prevent NAFLD in mice (Ritze et al., 2014, Xin et al., 2014), to our knowledge, this was the first study to suggest that NAFLD could be reduced by intragastric delivery of live *F. prausnitzii* cells. However, due to an extremely small sample population in this study, further interventions are needed in order to confirm our findings. Moreover, as the results from mouse studies are not straightforwardly applicable in human population, well-designed randomized controlled trials are required in order to comprehensively elucidate

the effects of *F. prausnitzii* on human metabolism. So far, an obvious challenge hampering the research on *F. prausnitzii*, as well as its possible therapeutic use, has been the extremely oxygen sensitive nature of the bacterium that has made it difficult to culture and preserve (Duncan et al., 2002, Martin et al., 2017). Strategies to increase the vitality of *F. prausnitzii* upon exposure to air are under constant development (Khan et al., 2014), possibly simplifying the culture conditions and making the production of probiotic supplements less challenging in the future. On the other hand, another potential approach to reveal the therapeutic impact of *F. prausnitzii* in human metabolism could be the identification and production of prebiotic dietary supplements that promote the growth of *F. prausnitzii* in the human GI tract. And in addition to the probiotic or prebiotic treatments, FMT has recently been proposed as one possible therapeutic method to improve obesity and associated metabolic disorders in humans (Vrieze et al., 2012, Gérard, 2016). As *F. prausnitzii* is one of the most abundant bacterial species in a healthy human gut (Suau et al., 2001, Miquel et al., 2014, Ferreira-Halder et al., 2017), it may have an important role in the overall effects of FMT. Research on this approach is still scarce, and method standardization and extensive randomized controlled trials are required in order to unveil the true capability of FMT to improve human metabolic health (Gérard, 2016). Propitiously, several ongoing randomized clinical trials have already been listed in the database of privately and publicly funded clinical studies (https://www.clinicaltrials.gov).

6.4 Limitations of the study

A small sample size is indisputably the main limitation in all subprojects of this study. Fecal samples from only four adult donors were analyzed in the methodological sub-study, and due to that no firm conclusions can be drawn from the acquired results. Further, the group sizes in the mouse studies were limited to 6 mice per study group, which hampered certain statistical analyses and may have thus affected the results and conclusions. In addition, the results from the mouse studies obviously cannot be generalized into human population. Although mice are a valuable study model, human and mice differ in both gut microbiota composition and overall metabolism (Takahashi et al., 2012). And as already discussed above, the analysis methods for gut microbiota composition analysis are developing extremely fast, and the 16S rRNA gene sequencing is already often supplemented or replaced by more powerful methodologies like shotgun metagenome sequencing. For this reason, certain findings of this study can already be considered as slightly outdated. In addition, it is a well-known fact that the 16S rRNA gene sequencing, like any molecular method, is prone to

biases (Kennedy et al., 2014, Santiago et al., 2014, Wagner Mackenzie et al., 2015), which may have affected the microbiota results of the original publications I and II. In future studies examining the role of gut microbiota modulation on host health, shotgun metagenome sequencing could provide a more profound picture on the composition and gene content of the microbes, whereas metatranscriptomics, metaproteomics and metabolomics could be applied to assess information about the functional changes in the gut.

In the mouse sub-studies, on the other hand, some drawbacks occurred that should be taken into account when planning the forthcoming rodent studies. For example, the HFD was so soft that even with chewing toys the teeth of the mice tended to overgrow, which affected the eating habits of the mice and potentially caused variation. In addition, there were certain misfortunes in the sample collection of the *E. cloacae* sub-study, and for instance 16S rRNA gene sequencing could not be included in the study because flaws in markings disallowed reliable identification of the sample tubes. Additionally, in future gut microbiota modification studies, the required amount of the inoculated bacteria, as well as an adequate administration schedule, should be more comprehensively determined in order to confirm a steady and constant colonization of the bacteria. And notably, bacterial treatment groups fed with chow diet should be included in the future studies in order to assess the effects of the bacterial treatment without synergetic effects with the HFD. For instance, in the *F. prausnitzii* study, it would have been interesting to see whether the fat mass of the mice would have also increased as a consequence of the intragastric bacterial administration, or whether it was solely due to the HFD. Further, in the *E. cloacae* study, it would have been important to assess whether the *E. cloacae* administration alone would have caused the liver damage observed in this study, or whether a heathier diet would have been adequate to protect the host from the harmful effects of the bacterial administration.

7 CONCLUSIONS

The methodological subproject of this study concludes that semi-automatic DNA extraction methods are practical and well-working options for the sample pre-processing in NGS studies. In addition, this study, along with previous studies (Bahl et al., 2012, Klindworth et al., 2013, Santiago et al., 2014, Kennedy et al., 2014, Brooks et al., 2015, Karst et al., 2018), highlights that comprehensive optimization of both pre-processing and analysis methods is required in order to produce reliable 16S rRNA gene sequencing results.

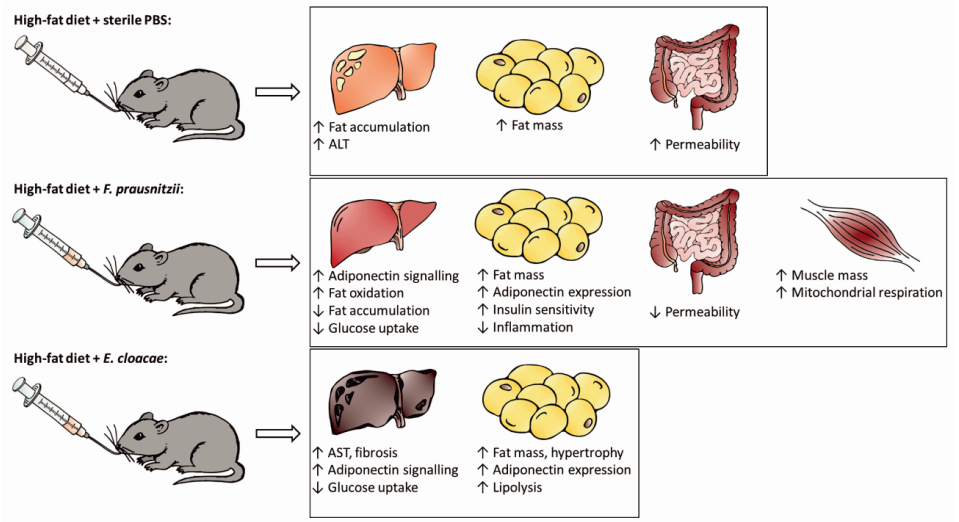

Figure 8: Summary of the main findings of studies II and III. As previously shown (Podrini et al., 2013), high-fat diet (HFD) induces hepatic fat accumulation in C57BL/6N mice. Gut microbiota modulation by intragastric *F. prausnitzii* administration seems to avert the detrimental effects of the HFD, whereas gut microbiota modulation by intragastric *E. cloacae* administration appears to induce liver damage. The effects of the HFD are presented as differences between the HFD controls and the chow controls, whereas the effects of the bacterial treatments are presented as differences between the bacterial treatment groups and the HFD controls. ↑ = increase; ↓ = decrease.

The results of the mouse studies suggest that *F. prausnitzii* treatment protects from the HFD-induced NAFLD in an experimental mouse model, whereas *E. cloacae* treatment causes liver damage (Figure 8). The underlying preventive mechanisms of the *F. prausnitzii* treatment likely involve increased fatty-acid oxidation and adiponectin signaling in liver and increased adiponectin expression in VAT. In addition, the *F. prausnitzii* -treatment seems to decrease the inflammation and increase the insulin sensitivity in the adipose tissues. By

contrast, the *E. cloacae* treatment tends to increase lipolysis and IR in the mouse adipose tissues, along with increasing SAT mass and the relative proportion of hypertrophic adipocytes.

In general, the findings of this study underline that the therapeutic potential of *F. prausnitzii* should be further studied. For example, humans with low abundance of *F. prausnitzii* could benefit from targeted gut microbiota modification. On the other hand, the results of this study emphasize the previously reported harmful effects of *E. cloacae*. In this study, the *E. cloacae* treatment caused liver damage without affecting the hepatic TAG content. Even though the results regarding the hepatic fat metabolism were partially contradictory to previous reports (Fei & Zhao, 2013), this study agrees with the previously demonstrated hypothesis that an increased proportion of *E. cloacae* in the GI tract may be harmful to the host health. The gained knowledge from this study can be utilized in the development of animal models in which the effects of the gut microbiota on host health and metabolism can be further assessed. One interesting future study goal could be to assess whether the onset of metabolic disorders and NAFLD in humans could be prevented by increasing the GI tract *F. prausnitzii* abundance or by strengthening the overall gut homeostasis via FMT. As long as the clinical applications of *F. prausnitzii* are hampered by its obligate anaerobic nature, another possible study goal could be to assess prebiotic dietary supplements that promote the growth of the bacterium. In fact, certain dietary supplements that promote the growth of *F. prausnitzii* have already been identified (Ramirez-Farias et al., 2008). Meanwhile, discovery of anti-inflammatory molecules secreted by *F. prausnitzii* may provide a step towards novel therapeutic applications. Research on this field is still in its infancy, but certain promising molecules have already been identified (Quévrain et al., 2016, Breyner et al., 2017).

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