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GUT MICROBIOTA AND METABOLIC DISORDERS

Eveliina Munukka

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To my family

ABSTRACT

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Gut microbiota and metabolic disorders

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Obesity and its co-morbidities, such as metabolic syndrome (MetS), non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes, have increased over the last few decades like an epidemic. So far the mechanisms of many metabolic diseases are not known in detail and currently there are not enough effective means to prevent and treat them. Several recent studies have shown that the unbalanced gut microbiota composition (GMC) and activity have an influence on the fat accumulation in the body. Further, it seems that the GMC of obese individuals differs from the lean.

The aim of this study was to investigate whether there are differences between the GMC of metabolically impaired overweight/obese (MetS group), metabolically healthy overweight/obese and normal-weight individuals. In addition, the mechanisms by which the gut bacteria as well as their specific structures, such as flagellin (FLG) that stimulates the Toll-like receptor 5 (TLR5) affect metabolism, were investigated both *in vivo* and *in vitro* in human adipocytes and hepatocytes.

The results of this study show that the abundance of certain gram-positive bacteria belonging to the *Clostridial* cluster XIV was higher in the MetS group subjects compared to their metabolically healthy overweight/obese and lean counterparts. Metabolically impaired subjects tended to also have a greater abundance of potentionally inflammatory Enterobacteria in their gut and thus seemed to have aberrant GMC. In addition, it was found that subjects with a high hepatic fat content (HHFC group) had less *Faecalibacterium prausnitzii* in their gut than individuals with low hepatic fat content. Further gene expression analysis revealed that the HHFC group also had increased inflammation cascades in their adipose tissue.

Additionally, metabolically impaired individuals displayed an increased expression of FLG-recognizing TLR5 in adipose tissue, and the TLR5 expression levels associated positively both with liver fat content and insulin resistance in humans. These changes in the adipose tissue may further contribute to the impaired metabolism observed, such as insulin resistance and dyslipidemia. *In vitro* -studies showed that the FLG-induced TLR5 activation in adipocytes enhanced the hepatic fat accumulation by decreasing insulin signaling and mitochondrial functions and increasing triglyceride synthesis due to increased glycerol secretion from adipocytes.

In conclusion, the findings of this study suggest that it may be possible that the novel prevention and personalized treatment strategies based on GM modulation will succesfully be developed for obesity and metabolic disorders in the future.

Key words: gut microbiota, obesity, metabolic disorders, liver fat, adipose tissue inflammation, TLR5, flagellin

TIIVISTELMÄ

EVELIINA MUNUKKA

Suolistomikrobisto ja metaboliset sairaudet

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Lihavuus ja sen liitännäissairaudet kuten metabolinen oireyhtymä (MetS), ei-alkoholiperäinen rasvamaksa (NAFLD) sekä tyypin 2 diabetes ovat lisääntyneet viime vuosikymmenien aikana epidemian lailla. Toistaiseksi monien metabolisten sairauksien syntymekanismeja ei tunneta yksityiskohtaisesti eikä niiden ennaltaehkäisyyn ja hoitoon myöskään ole olemassa tarpeeksi tehokkaita keinoja. Useat viimeaikaiset tutkimukset ovat osoittaneet, että ylipainolla ja aineenvaihdunnallisilla sairauksilla on yhteys epätasapainoiseen eli dysbioottiseen suolistomikrobistoon ja, että esimerkiksi normaali- ja ylipainoisten yksilöiden suolistomikrobiston koostumukset eroavat toisistaan.

Tämän väitöskirjatutkimuksen tarkoituksena oli selvittää mahdollisia suolistomikrobistoprofiiliin liittyviä eroavaisuuksia aineenvaihdunnallisesti terveiden ylipainoisten ja normaalipainoisten sekä metabolisen oireyhtymän kriteerit täyttävien aikuisten välillä. Lisäksi selvitettiin sekä ihmisaineistossa että rasva- ja maksasolumalleilla niitä mekanismeja, joilla ihmisen suolistomikrobistoon kuuluvat bakteerit sekä niiden rakenneosat kuten erityisesti tollin kaltaista reseptoria 5 (TLR5) stimuloiva flagelliini vaikuttavat ihmisen aineenvaihduntaan.

Tulokset osoittivat, että ylipainoisten, häiriintyneen aineenvaihdunnan omaavien henkilöiden (ns. MetS-ryhmä) suolistomikrobisto erosi sekä terveistä yli- että normaalipainoisista henkilöistä. Erityisesti MetS-ryhmällä oli enemmän tiettyjä Klostridi XIV-ryhmän bakteereja sekä myös enemmän mahdollisesti tulehdusta aiheuttavia Enterobakteereja. Lisäksi havaittiin, että henkilöillä, joilla oli korkea maksan rasvapitoisuus (HHFC-ryhmä) oli vähemmän *Faecalibacterium prausnitzii* –bakteeria kuin alhaisen maksan rasvapitoisuuden omaavilla henkilöillä. Lisäksi HHFC-ryhmällä oli käynnissä enemmän tulehdusprosesseja rasvakudoksessaan. Flagelliinia tunnistavaa TLR5 ilmentyi enemmän häiriintyneen aineenvaihdunnan omaavien henkilöiden rasvakudoksessa, ja lisäksi ihmisissä TLR5:llä havaittiin voimakas positiivinen korrelaatio sekä maksan rasvaprosentin että insuliiniresistenssin kanssa. Solukokeissa edelleen havaittiin, että mahdollisesti suolistobakteereista peräisin olevalla flagelliinilla sekä sen havaitsevalla TLR5:llä voi olla rooli rasvakudoksen tulehdusreaktioissa sekä maksan epänormaalissa rasvoittumisessa.

Väitöskirjassa saavutetut tulokset voivat mahdollisesti toimia tulevaisuudessa lähtökohtana uudentyyppisten metabolisten sairauksien ehkäisy- ja hoitokeinojen kehitystyössä, joissa mikrobistokoostumusta ja -toiminnallisuutta muokkaamalla pyritään ohjaamaan elimistön aineenvaihduntaa suotuisaan suuntaan. Tulevaisuudessa erilaiset bakteeriterapiat voivatkin olla osa yksilön lähtökohdat huomioonottavaa, personoitua lääketiedettä.

Avainsanat: suolistomikrobisto, ylipaino, metaboliset sairaudet, tulehtunut rasvakudos, rasvamaksa, TLR5, flagelliini

TABLE OF CONTENTS

SISÄLTÖ

ABSTRACT **4**
TIIVISTELMÄ **5**
ABBREVIATIONS **9**
LIST OF ORIGINAL PUBLICATIONS **11**

1. INTRODUCTION **12**

2. REVIEW OF THE LITERATURE **14**
2.1 Gut microbiota and health 14
2.1.1 The important bacterial players in GI tract 18
2.1.1.1 Firmicutes: Clostridium cluster IV and Faecalibacterium prausnitzii, Clostridium cluster XIV and Eubacterium, Ruminococcus 18
2.1.1.2 Actinobacteria: Bifidobacterium sp., Atopobium cluster 20
2.1.1.3 Bacteroidetes: Bacteroides spp. and Prevotella 21
2.1.1.4 γ-Proteobacteria: family Enterobacteriaceae 23
2.1.1.5 Akkermansia muciniphila 24
2.1.1.6 Non-bacterial players 24
2.1.2 Development and stability of gut microbiota 25
2.1.3 Healthy gut microbiota, core microbiome and enterotypes 26
2.1.4 Methods to study gut microbiota composition 28
2.1.4.1 Sampling and storage 29
2.1.4.2 Molecular methods based on the sequence variability of 16S rRNA gene 30
2.2 Factors that determine and shape the composition and function of GM 33
2.2.1 Gender 34
2.2.2 Diet and gut microbiota 35
2.2.2.1 Western diet and GM *36*
2.2.3 The effect of antibiotics on GM composition 37
2.3 Intestinal immune system and gut microbiota are dependent on each other 38
2.3.1 Leaky gut and dysbiosis 39
2.3.2 Toll-like receptors 41
2.3.2.1 Toll-like receptor 2 (TLR2) 42
2.3.2.2 Toll-like receptor 4 (TLR4) 42
2.3.2.3 Toll-like receptor 5 (TLR5) 43

2.4 Gut microbiota, obesity and metabolic disorders 44
2.4.1 Obesity and gut microbiota 46
2.4.1.1 Examples of bacterial groups and species that has been associated with obesity and increased body fat accumulation ...47
2.4.1.2 Examples of bacterial groups and species that has been associated with good metabolic health 48
2.4.1.3 The effect of antibiotics on overall GMC and adiposity 50
2.4.1.4 The role of gut microbiota metabolites 50
2.4.2 Gut microbiota, metabolic syndrome and type 2 diabetes 52
2.4.2.1 Gut microbiota and adipose tissue inflammation 53
2.4.2.2 Endocannabinoid system (eCB) in regulation of gut-adipose tissue axis 54
2.4.2.3 Gut microbiota in type 2 diabetes 54
2.4.3 Excessive hepatic fat accumulation and gut microbiota 56
2.4.3.1 Proposed mechanisms of gut-liver axis in the pathogenesis of NALFD 57
2.4.4 Cardiovascular diseases (CVD) and atherosclerosis 58
2.4.5 Gut microbiota modification as a treatment strategy in metabolic diseases 59

3. AIMS OF THE STUDY 62

4. MATERIALS AND METHODS 63

4.1 Human study design 63
4.1.1 Characteristics of human study subjects 64
4.1.2 Anthropometrics, body composition and diet (I-IV) 65
4.1.3 Blood samples (I-IV) 66
4.1.4 Gut microbiota profiling of fecal samples (I-III) 66
4.1.5 Experiments performed with the adipose tissue biopsies (II-IV) 67
4.1.5.1 Microarray experiments 67
4.1.6 Cell lines and cell culture experiments (III, IV) 68
4.1.6.1 Imaging of the adipocytes by immunofluorescence (III,IV) 69
4.1.6.2 Western blot analysis 69
4.1.6.3 RT qPCR 69
4.1.6.4 Neutral lipid, Reactive Oxygen Species (ROS) and glycerol measurements in hepatocytes 70
4.1.7 Statistics 70
4.2 Results and discussion 71
4.2.1 Overall description of the GMC 71

4.2.2 Subjects with metabolic disorders have a significantly different gut microbiota profile compared to their metabolically healthy counterparts (I) 73
4.2.3 Aberrant gut microbiota associates with higher liver fat content in human subjects simultaneously with adipose tissue inflammation (II) ... 75
4.2.3.1 Adipose tissue gene expression in HHFC individuals and its association with GMC 76
4.2.4 Over-expression of Toll-like receptor 5 in subcutaneous adipose tissue associated with obesity, metabolic changes and dysbiotic gut microbiota composition in humans (III) 77
4.2.4.1 The clinical manifestations, metabolism and GMC in H-TLR group 79
4.2.5 *In vitro* studies in cultured human adipocytes: FLG induces functional changes in the adipocyte metabolism (III) 80
4.2.5.1 Insulin signaling studies 83
4.2.6 Adipose tissue *TLR5* mRNA correlates with hepatic fat content and insulin sensitivity in humans (IV) 83
4.2.7 The effects of flagellin on hepatocyte fat accumulation and metabolism (IV) 84
4.2.7.1 The effects of FLG and LPS on insulin signaling in hepatocytes (IV) 86
4.2.7.2 The effects of FLG and LPS treatments on inflammation and mitochondrial respiratory chain complex subunits in hepatocytes (IV) 87
4.2.8 Limitations of the study 88

5. SUMMARY AND CONCLUSIONS 90

ACKNOWLEDGEMENTS 92

REFERENCES 94

ORIGINAL PUBLICATIONS I–IV 113

ABBREVIATIONS

ANCOVA	analysis of covariance
ANOVA	analysis of variance
Bacto group	*Bacteroides-Prevotella-Porphyromonas* group
BMI	body mass index
BP	blood pressure
CCM	conditioned culture media
CONV	conventionally raised
CVD	cardiovascular disease
DC	dendritic cell
DNA	deoxyribonucleic acid
DXA	dual-energy X-ray absorptiometry
EDTA	ethylenediamine tetra-acetic acid
Erec	*Eubacterium rectale – Clostridium coccoides* group, i.e. Clostridial cluster XIVa
F/B ratio	*Firmicutes*-to-*Bacteroidetes* ratio
FCM	flow cytometry
FLG	flagellin
FISH	fluorescence *in situ* hybridization
FM	fat mass
FM %	fat mass percentage
Fprau	*Faecalibacterium prausnitzii*
G+C	guanine + cytosine
GF	germ free
GI	gastrointestinal
GIT	gastrointestinal tract
GM	gut microbiota
GMC	gut microbiota composition
HDL	high density lipoprotein
HFA/HFC	hepatic fat accumulation/content
HFD	high-fat diet
^{1}H MRS	proton magnetic resonance spectroscopy
HOMA	homeostatic model assessment
IBD/ IBS	inflammatory bowel disease/syndrome
IL	interleukin
IR	insulin resistance
IIS	intestinal immune system
KO	knock out

LDL	low density lipoprotein
LM	lean mass
LPS	lipopolysaccharide
MAMP	microbial associated molecular patterns
MMP	Matrix metalloprotease
MBI	Microbial Balance Index
MD	metabolic disorder
MetS	metabolic syndrome
NAFLD	non-alcoholic fatty liver disease
NF-κB	Nuclear factor kappa B
NGS	next generation sequencing
NLR	Nod-like receptor
o/n	over night
OTU	operational taxonomic unit
PBS	phosphate buffered saline
PRR	Pattern recognition receptor
PCR	polymerase chain reaction
qPCR	quantitative PCR
rRNA	ribosomal RNA
RT	room temperature
SCFA	short chain fatty acids
SD	standard deviation
SDS	sodium dodecyl sulphate
SLGI	systemic low grade inflammation
T2D	Type 2 diabetes
TJ	tight junction
TLR	Toll-like receptor
TNF	tumour necrosis factor
T_{reg}	regulatory T cell
TG	triglyceride
WC	waist circumference
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

I. Munukka Eveliina, Wiklund Petri, Pekkala Satu, Völgyi Esther, Xu Leiting, Cheng Shumei, Lyytikäinen Arja, Marjomäki Varpu, Alen Markku, Vaahtovuo Jussi, Keinänen-Kiukaanniemi Sirkka and Cheng Sulin. Women with and without metabolic disorder differ in their gut microbiota composition. Obesity (Silver Spring). 2012; 20(5): 1082-7.

II. Munukka Eveliina*, Pekkala Satu*, Wiklund Petri, Rasool Omid, Borra Ronald, Kong Lingjia, Ojanen Xiaowei, Cheng Shumei, Roos Christophe, Tuomela Soile, Alen Markku, Lahesmaa Riitta and Cheng Sulin. Gut-adipose tissue axis in hepatic fat accumulation in humans. J Hepatology. 2014; 61(1): 132-8.

III. Pekkala Satu*, Munukka Eveliina*, Kong Lingjia, Pöllänen Eija, Autio Reija, Roos Christophe, Wiklund Petri, Fischer-Posovszky Pamela, Wabitsch Martin, Alen Markku, Huovinen Pentti and Cheng Sulin. Toll-like receptor 5 in metabolic disorders-the role of gut microbiota and adipose tissue inflammation. Obesity (Silver Spring). 2015; 23(3): 581-90. * Equal contribution

IV. Munukka Eveliina, Wiklund Petri, Partanen Tiina, Välimäki Sakari, Pöllänen Eija, Lehti Maarit, Fischer-Posovszky Pamela, Wabitsch Martin, Cheng Sulin, Huovinen Pentti and Pekkala Satu. Adipocytes as a link between gut microbiota-derived flagellin and hepatocyte fat accumulation. *Manuscript submitted.*

1. INTRODUCTION

The incidence of obesity and metabolic disorders are reaching epidemic proportions all over the world (Caballero 2007, IDF Diabetes Atlas 2015, Pereira *et al.* 2015). Today, obesity and related chronic comorbidities such as insulin resistance (IR), type II diabetes (T2D), hypertension, fatty liver and cardiovascular diseases (CVD) are considered to be the major threats for the general health of the human population (Ng *et al.* 2014). Today over 500 million people worldwide are obese, i.e., their body mass index exceed 30 kg/m^2 (Swinburn *et al.* 2011) and there are nearly 60 million individuals with diabetes currently in Europe (IDF Diabetes Atlas 2015). The problem does not concern only adults in western world but is affecting developing countries and alarmingly also children (Swinburn *et al.* 2011, IDF Diabetes Atlas 2015).

The excess gain of the body fat mass (FM) arises from the complex interactions between the genetic and environmental factors that evoke the continuous imbalance in the energy intake and expenditure (Hill 2006, Lean *et al.* 2006). The epidemic rise observed in the developed countries has been explained by the excessive consumption of the energy-dense food combined with an inactive lifestyle (Hill 2006, Lean *et al.* 2006). However despite the constantly growing scientific knowledge, exact underlying mechanisms for the pathophysiology of the obesity-related disorders are not fully known. Although more and more attention is given to the energy content of the diet and too low daily physical activity of the individuals, the average percentage of fat tissue in human bodies is rising all around the world (Swinburn *et al.* 2011). Therefore current high incidence rate of obesity and metabolic disorders cannot be solely attributed to above-mentioned factors (Dhurandhar *et al.* 2014). In addition, some individuals seem to be more susceptible to weight gain and obesity than others despite similar lifestyle (Dhurandhar *et al.* 2014). Thus it seems that other predisposing factors than genetics and wrong nutritional choices and lifestyle must affect energy and fat metabolism of human beings.

Obesity is often accompanied with insulin resistance (IR) that is a state of impaired insulin function and systemic low-grade inflammation (SLGI), in which a great number of different cytokines and adopokines are produced by immune cells (IC) and adipose tissue itself (Hotamisligil 2003, Reaven 2005, Guida & Venema 2015). This predisposes the host to the metabolic syndrome (MetS), a state that is described by the co-occurrence of several cardio-metabolic risk factors such as increased abdominal obesity, elevated blood pressure (BP) levels, hyperglycemia and dyslipidemia, i.e., high serum triglyceride (TG), as well as low high-density (HDL) cholesterol level (Alberti *et al.* 2005). Prolonged IR may further lead to the development of type 2 diabetes (T2D), which is currently affecting nearly 10 % of the adult population all over the world and rates of the incidence are constantly increasing (IDF Diabetes Atlas 2015).

Recent accumulative evidence suggests that microbes colonizing the gastrointestinal (GI) tract are key factors that influence on the metabolism and energy balance of the host and several mechanisms have been identified how gut microbiota (GM) may act as a link between the inflammation and metabolic complications such as obesity (Bäckhed *et al.* 2004, Ley *et al.* 2005, Turnbaugh *et al.* 2006, Cani *et al.* 2007a, Turnbaugh *et al.* 2009a). Therefore, today's medicine no longer overlooks the role of our microbial counterparts in the metabolic health, and GM is considered to be an important metabolic organ residing within the host (Tremalori & Bäckhed *et al.* 2012, Karlsson *et al.* 2013, Lankelma *et al.* 2015, Marchesi *et al.* 2015). During the past decade several studies have acknowledged the role of GM in various diseased states such as acute gastroenterological infections, inflammatory bowel disease (IBD) and allergy (reviewed in Cho & Blaser 2012, Marchesi *et al.* 2015). Knowledge on the importance of GM for human health has rapidly increased through the exploitation of modern, high-throughput, next generation sequencing (NGS) and systems approaches in combination with the germ-free (GF) animals as host models (Cho & Blaser 2012, Salonen 2013). However, the mechanisms behind the assumptions have still not been clarified in detail (reviewed in Zhang *et al.* 2010, Rosenbaum *et al.* 2015).

GM affects the harvest and storage of the energy obtained from the diet as well as its expenditure and thus may act as a novel, intriguing factor contributing to the obesity epidemic (reviewed in Sekirov *et al.* 2010, Krajmalnik-Brown *et al.* 2012, Tremalori & Bäckhed 2012, Villanueva-Millán *et al.* 2015). Gut microbes are capable of influencing the levels of blood glucose, magnitude of the fat storage in body and how host responds to the satiety hormones. Thus a wrong assembly of gut microbes may set the stage for obesity, chronic inflammation and metabolic disorders (Tremalori & Bäckhed 2012, Sommer & Bäckhed 2013, Allin *et al.* 2015, Delzenne *et al.* 2015). Therefore the GM modulation via personalized bacterial therapy or dietary concepts provides novel and cost-effective strategies for the management of obesity and metabolic diseases in the future (Delzenne *et al.* 2011a, Xiao & Zhao 2014). However, to date most of the data is obtained from the studies conducted in GF animal models, so the direct translation of findings to human diseases is challenging (Salonen 2013). In addition, studies reporting the causal role of GM composition (GMC) in metabolic diseases in humans still remain scarce and the exact mechanisms are not well understood (Zhao 2013).

2. REVIEW OF THE LITERATURE

2.1 Gut microbiota and health

Humans serve as a walking culture medium for heavy loads of microbial organisms. We have co-evolved with our prokaryotic companions (Ley *et al.* 2008). Microbes live in and on the human body, and all of our exposed body sites such as gastrointestinal (GI), urogenital, nasal and respiratory tract, skin and mouth are occupied by the dense microbial communities that all together constitute microbiota (Savage *et al.* 1977, Ley *et al.* 2008, Sekirov *et al.* 2010). Since microbial cells outnumber our cells roughly ten times, humans can be actually referred to microbial superorganisms (Savage 1977, Gill *et al.* 2006, Costello *et al.* 2009, Sekirov *et al.* 2010). Nowadays GM is considered to be more or less essential and beneficial companion to host and it is seen as an organ within an organ (Bäckhed *et al.* 2005, O'Hara & Shanahan 2006, Sommer & Bäckhed 2013, Evans *et al*. 2013, Hollister *et al.* 2014). In addition, GM has been suspected to play a role in the etiopathogenesis of various chronic diseases such as allergy, type I diabetes (T1D) and even autism and depression (reviewed in Sekirov *et al.* 2010, Cho & Blaser 2012, de Vos & de Vos 2012, Meri & de Vos 2015).

The exceptionally complicated and abundant microbial community inhabits the GI tract, the area of which equals to the size of a tennis field (Savage *et al.* 1977, Sekirov *et al.* 2010). It is estimated that GM consists of even 10^{14} microorganisms and the colon encompasses the densest bacterial ecosystem on Earth reaching as much as 10^{12}/g of gut contents (Figure 1) thus gut serves as a venue for the important host-microbial interactions (Eckburg *et al.* 2005, O'Hara & Shanahan 2006, Sekirov *et al.* 2010, Clemente *et al.* 2012, Ursell *et al.* 2012, Rajilic-Stojanovic & de Vos 2014). GM possesses a great phylogenetic richness and bacteria outnumber other microbial habitats in GI tract (Savage 1977, Eckburg *et al.* 2005, Rajlic-Stojanovic *et al.* 2007, Costello *et al.* 2009, Rajilic-Stojanovic & de Vos 2014). Each individual harbors an unique GM composition (GMC), which is heterogenic spatially across the gut and longitudinally across the length of the GI tract (Figure 1) and each compartment harbors distinct microbial ecosystem (Ursell *et al.* 2012). The individuality of GM structure arises from the fact that many host-related factors such as genetics, age, health status and gender together with the various environmental factors such as geography, diet, hygiene level, medications and toxins have an influence on its composition (Sekirov *et al.* 2010, Yatsunenko *et al.* 2012, Ursell *et al.* 2012, Xu & Knight 2015).

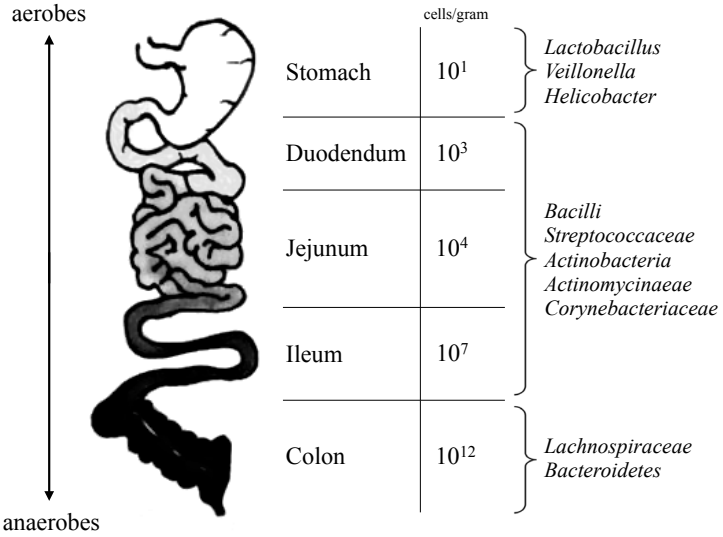

Figure 1. Variations in bacterial numbers and composition across the length of the gut**.** Modified from (Sekirov *et al.* 2010). Published with the permission of Anniina Rintala, MSc. who drew the figure.

Recent studies utilizing massive, parallel sequencing technologies have estimated that the number of different bacterial species residing in gut may exceed over 1,000 including representatives from at least ten bacterial phyla (Eckburg *et al.* 2005, Turnbaugh *et al.* 2009a, Qin *et al.* 2010, Morgan *et al.* 2013, Rajilic-Stojanovic & de Vos 2014, Sankar *et al.* 2015). Besides bacteria, *Archaea*, viruses (mainly bacterial phages) and eukaryotes can be found in gut as minor microbial representatives (Eckburg *et al.* 2005, Parfrey *et al.* 2014). Still to date it is estimated that less than 50 percent of human GM is characterized thus its diversity and function are far from fully known (Lagier *et al.* 2012). The overall composition of human GM is represented in Table 1.

Over 90 percent of gut bacteria belong either to the gram-positive *Firmicutes* (*Clostridia, Bacilli* and *Mollicutes*)*,* the gram-negative *Bacteroidetes* (genus *Bacteroides*, *Porphyromonas* and *Prevotella*) or the gram-positive *Actinobacteria* (genus *Bifidobacterium* and *Collinsella*) phylum (Eckburg *et al.* 2005, Wilson 2005, Qin *et al.* 2010). In the colon, the three most numerous groups within these three phyla are *Bacteroides, Clostridium* cluster XIVa aka *C. coccoides* group, and *Clostridium* cluster IV aka *C. leptum* group that all are strict anaerobes (Mahowald *et al.* 2009, Sekirov *et al.* 2010). Other important genera within *Firmicutes* that have been isolated from the GI tract are *Lactobacillus, Peptostreptococcus, Streptococcus* and *Veillonella.* In addition, members of subdominant bacterial phyla such as *Fusobacteria, Proteobacteria* and mucus-colonizing *Verrucomicrobia* (consisting mainly of one important species, *Akkermansia muciniphila*) are important representatives in the overall, balanced GM composition that affect host's health (Eckburg *et al.* 2005, Qin *et al.* 2010, Lozupone *et al.* 2012, Lopetuso *et al.* 2013).

Table 1. Overview of the composition of the human colon microbiota. (reviewed in Rajilic-Stojanovic & de Vos 2014).

PHYLUM	GROUP/GENUS	IMPORTANT SPECIES	FUNCTIONS	REFS
Bacteroidetes	*Bacteroides*	*B. thetaiotaomicron, B. distasonis, B. vulgatus, B. ovatus, B. fragilis*	polysaccharide degradation, some harbor proteolytic activity	Eckburg *et al.* 2005, Turnbaugh *et al.* 2009.
	Parabacteroides			
	Porphyromonas	*P. gingivalis*		Wu *et al.* 2011
	Prevotella	*P. melaninogenica*	propionate production by some species	
Firmicutes	*Clostridium* cluster XIVa/*Eubacterium, Clostridium, Roseburia, Ruminococcus, Butyrifibrio*	*E. rectale, E. limosum, Cl. saccharolyticum, R.intestinalis, R. hominis, R.obeum, R.gnavus, B. fibrisolvens*	polysaccharide degradation, butyrate production by some species	Eckburg *et al.* 2005, Turnbaugh *et al.* 2006, 2009
	Clostridium cluster IV/*Faecalibacterium*	*F.prausnitzii*	butyrate production, relieves inflammation	Duncan *et al.* 2002, Sokol *et al.* 2008
		R.bromi		Ze *et al.* 2012
	Lactobacillus	*L. acidophilus, L. casei, L. plantarum, L.brevis*	beneficial properties for the host	Vaughan *et al.* 2002, Tannock, 2004
Actinobacteria	*Atopobium* cluster	*Collinsella aerofaciens*		Gupta *et al.* 2013
	Bifidobacterium	*B. adolescentis, B. infantis, B. breve*	beneficial properties for the host	Ventura *et al*. 2007a
γ-Proteobacteria	*Escherichia*	*E. coli*	proinflammatory, potential pathogen	Eckburg *et al.* 2005
	Enterobacteria	*E. cloacae*	proinflammatory	Fei & Zhao 2013
δ-Proteobacteria	*Bilophila*	*B. wadsworthia*	utilizes taurin, generates hydrogen sulphide, causes irritation and discomfort, colitis	Devkota *et al.* 2012
		Desulfovibrio spp.	sulfate-reducer, inhibition of butyrate oxidation	Fite *et al*. 2004
Fusobacteria	*Fusobacterium*	*F. nucleatum*	proinflammatory	Wu *et al.* 2011
Verrucomicrobia	*Akkermansia*	*A. muciniphila*	mucin degrader	Derrien *et al.* 2004

Gut microbiome is a collective of all microbial genomes and genes within the human GM (Pflughoeft & Versalovic 2012, Lepage *et al.* 2013, Morgan *et al.* 2013). The first metagenomic analysis of human fecal samples was published almost a decade ago (Gill *et al.* 2006). Few years later a reference gene catalogue (3.3 million genes) was generated from 124 human fecal samples as a result of European Metagenomics of the

Human Intestinal Tract (MetaHIT) project (Qin *et al.* 2010). Current catalogues suggest that gut microbiome is a 100-fold larger gene pool than host's genome (Qin *et al.* 2010, Morgan *et al.* 2013). Global Gut Microbial Gene catalogue that combined the data from four human GM studies conducted in three different continents (Europe, North America and Asia, $n = 782$) was published just recently (Karlsson *et al.* 2014). However, despite the intensive approaches the diversity of GM remains at least partly unsolved.

The recent description of the whole gene catalogues of the GM has revealed that it is enriched with the genes encoding various biochemical pathways that are essential for human life. GM contains a great variety of different genes involved in the host's metabolism and physiology, and thus provides the host with such functions that its own genome is lacking (Turnbaugh *et al.* 2006, Gill *et al.* 2006, Turnbaugh *et al.* 2009, Qin *et al.* 2010, Li *et al.* 2014). These include the breakdown of complex dietary polysaccharides, the fermentation of glycans, the biosynthesis of amino acids and vitamins such as B_{12} and K, the biotransformation of bile acids, the detoxification of xenobiotics and methanogenesis (Gill *et al.* 2006, Turnbaugh & Gordon 2009, Qin *et al.* 2010, Griffin *et al.* 2015). As a result of microbial actions a myriad of microbial metabolites such as short-chain fatty acids (SCFA) are produced and further participate in the regulation of the host signaling pathways and gene expression (Paparo *et al*. 2014). Overall, the GM serves as an additional organ that participates in the development and functions of other organs of the host (Evans *et al.* 2013) as well as maintains and regulates the intestinal barrier function (Walker & Lawley 2013) and participates in the synthesis, digestion and metabolism of nutrients derived from food (Sekirov *et al.* 2010, Scaldaferri *et al*. 2012, Tremaroli & Bäckhed 2012, Sommer & Bäckhed 2013, Hollister *et al.* 2014). Some features of the microbiome are shared among humans and at the same time others are highly personalized (Qin *et al.* 2010).

A finetuned, bidirectional connection exists between the bacteria and the immune cells of the host in the gut, and GM contributes to the development, maintenance and regulation of host's immune system (Hooper & Gordon 2001, Sekirov *et al.* 2010, Hooper *et al.* 2012). GM together with the intestinal epithelial and mucous layer makes up an anatomical and functional barrier that separates the intestinal contents from the other tissues of the host. The submucosal layer that lies in the vicinity of gut lumen in the small intestine, namely gut-associated lymphoid tissue (GALT), serves as an abundant reservoir of the immunocytes such as dendritic (DC) and M-cells (Scaldaferri *et al.* 2012). Early life is crucial time for the development of the balanced host-microbe symbiosis since GM and immune system develop collaterally during the infancy. Success in this multifaceted development has further a major impact on health throughout life (Martin *et al.* 2010, Bengmark 2013, Wopereis *et al.* 2014).

This literature review focuses on the most important gut bacterial groups, genera and species that possess enzymatic activities that regulate metabolic functions of the host,

i.e., participate in the host's metabolism. These gut bacteria and their relevance to human health and disease are presented in Table 1.

2.1.1 The important bacterial players in GI tract

2.1.1.1 Firmicutes: Clostridium cluster IV and Faecalibacterium prausnitzii, Clostridium cluster XIV and Eubacterium, Ruminococcus

Firmicutes is the most abundant phylum in the human GI tract (Rajilic-Stojanovic & de Vos 2014). Two distinct gram-positive, spore-forming classes *Bacilli* and *Clostridia*, both usually containing low G+C % in their genomic DNA, are the most dominate representatives of this phylum (Wilson 2005, Rajilic-Stojanovic & de Vos 2014). The taxonomic classification of the *Firmicutes* phylum has been quite a challenging task for microbiologists, and for example not all members express a gram-positive staining pattern (Stackebrandt *et al.* 1999, Yutin & Galperin 2013, Rajilic-Stojanovic & de Vos 2014). Due to the enormous diversity, a lot of phylogenetic intermixing between *Clostridium* and other genera existed during the era of traditional culture-based taxonomy, and still their systematics are in a continuous flux (Yutin & Galperin 2013). Currently *Clostridiales* are divided into 19 distinct clusters (I-XIX) based on their location in the 16S rRNA gene-based phylogenetic tree. Distinguished spore-forming pathogens such as *C. botulinum, C. perfringens and C. tetani* belong to cluster I that is referred also as true *Clostridium* spp. i.e. *C. sensu stricto.* The presence of these *C. sensu stricto* species in GIT is usually considered as a biomarker of disturbed microbiota (Stackebrandt *et al.* 1999). Important intestinal pathogen *C. difficile* belongs to the cluster XI (Gupta & Gao 2009). However, most *Clostridium* spp. maintain a commensal or symbiotic relationship with the host (Rajilic-Stojanovic & de Vos 2014) .

The most substantial *Clostridium* clusters habituating the large intestine are IV and XIVa. Together they make up an intrinsic portion of the total GM (Mahowald *et al.* 2009, Lopetuso *et al.* 2013, Rajilic-Stojanovic & de Vos 2014). Bacteria within these clusters are found to be present mainly in mammalian GIT (Meehan & Beiko 2014). Cluster XIVa mainly consists of the members of *Lachnospiraceae* and IV of the *Ruminococcaceae* family (Tap *et al*. 2009, Rajilic-Stojanovic & de Vos 2014). They both include species capable of butyrate production, which is the preferred energy source for the colonocytes thus protecting the distal gut from various diseases (Pryde *et al.* 2002, Hamer *et al.* 2008). In addition, *Clostridium* IV and XIVa species are important for the maintenance of gut immune homeostasis and the so-called gut-brain axis by stimulating the production of regulatory cells (T_{REG}) in the colon and free catecholamines in the gut lumen as was demonstrated in a series of studies that utilized a cocktail of altogether 46 important gut *Clostridium* IV and XIVa species (Atarashi *et al.* 2011, Asano *et al.* 2012).

Clostridium cluster IV

Clostridium cluster IV aka *C. leptum* group includes for example *Clostridium, Faecalibacterium, Ruminococcus and Subdoligranulum* species that all are able to utilize various different carbohydrates as precursors for the fermentation. As a result, at least acetate, butyrate, propionate, valerate, CO_2 and H_2 are produced depending on the species (Miquel *et al.* 2014). One of the most important members of the cluster IV is *Faecalibacterium prausnitzii* that has recently been the focus of intensive research due to its health-promoting properties (Duncan *et al.* 2002, Sokol *et al.* 2008, Miquel *et al.* 2015). *F. prausnitzii* is a non-motile and non-sporeforming rod that utilizes at least glucose, fructose, pectin and fructo-oligosaccharides as energy source and produces butyrate, formate, D-lactate and CO_2 as a result of the fermentation process (Duncan *et al.* 2002, Lopez-Silas *et al.* 2012). Current taxonomical knowledge places *F. prausnitzii* into the *Clostridium* cluster IV within the *Ruminococcaceae* family (Duncan *et al.* 2002). Interestingly, *F. prausnitzii* was originally referred to *Fusobacterium prausnitzii* due to its gram-negative staining pattern (Duncan *et al.* 2002). However, further physiological and phylogenetic analysis based on 16S rRNA gene sequencing has revealed that it is only distantly related to *Fusobacteria* and is more closely related to members of *Clostridium* cluster IV (Duncan *et al.* 2002).

F. prausnitzii is one of the most abundant bacterial species in a healthy human gut (Holdeman *et al.* 1976, Duncan *et al.* 2002) and has recently been suggested to serve as a biomarker for a healthy gut (Li *et al.* 2008, Sokol *et al.* 2008, Miquel *et al.* 2014). For example, various studies have demonstrated that *F. prausnitzii* possesses apparent anti-inflammatory effects, and has a capability to suppress inflammation presumably via metabolites that are secreted during the fermentation process (Sokol *et al.* 2008, Sokol *et al.* 2009, Martin *et al.* 2015, Miquel *et al.* 2015, Quévrain *et al.* 2015). Both the bacteria itself and its culture supernatant have been found to induce an increased IL-10 and decreased IL-12 and tumor necrosis factor α (TNFα) production *in vivo* (Sokol *et al.* 2008). In addition, a decreased amount of the fecal *F. prausnitzii* has been reported in subjects with various intestinal diseases, such as IBD (both ulcerative colitis and Crohn's disease) (Sokol *et al.* 2008, Sokol *et al.* 2009) as well as colon cancer (Kang *et al.* 2010). Recently, low levels of *F. prausnitzii* were also associated with reduced bacterial diversity and richness in the gut as well as with the increased adiposity in obese/metabolically impaired subjects (Le Chatelier *et al.* 2013). In addition, Li *et al.* reported that the fecal level of *F. prausnitzii* was linked to the several urinary metabolites such as dimethylamine, taurine, lactate, glycine, 2-hydroxyisobutyrate, glycolate, 3,5-hydroxylbenzoate and 3-aminoisobutyrate indicating that it really is a functionally active member of the GM (Li *et al.* 2008). However, mechanisms behind the possible health effects of *F. prausnitzii* are still uncertain and under constant research.

Clostridium cluster XIVa

Clostridium cluster XIVa members belong to the *Lachnospiraceae* family and include species from *Blautia*, *Butyrivibrio*, *Clostridium, Coprococcus, Dorea, Eubacterium*, *Roseburia* and *Ruminococcus* that all are prominent members of the healthy, balanced GM (Hold *et al.* 2002, Lopetuso *et al.* 2013, Rajilic-Stojanovic & de Vos 2014). Many representatives of this cluster are efficient producers of butyrate that serves as a major energy source for the gut epithelial cells, has anti-inflammatory and anticarcinogenic properties and recently has also been associated with benign energy metabolism (Hamer *et al.* 2008, de Vadder *et al.* 2014). Low levels of the butyrate-producing *Lachnospiraceae* have been associated with inflammatory intestinal diseases and colorectal cancer (Wang *et al.* 2012, Rajilic-Stojanovic *et al.* 2013). The importance of *Lachnospiraceae* is further emphasized by the fact that they are among the first polysaccharide-degrading bacteria that inhabit the infant gut and thus are important drivers in the development of a functional intestinal immune system (Lopetuso *et al.* 2013). Recently some members within the *Clostridium* cluster XIV have been also linked to the increased body adiposity (Turnbaugh *et al.* 2006, Cho *et al.* 2012, Duca *et al.* 2014)

Eubacterium rectale is a notable butyrate-producer within the cluster XIVa and it is one of the most abundant singular species that colonizes the human gut (Eckburg *et al.* 2005, Aminov *et al.* 2006, Louis & Flint 2009). *E. rectale* strains are able to utilize at least glucose, sucrose, fructose, cellobiose, raffinose, soluble starch and inulin as an energy source, and butyrate, lactate, formate and H_2 are major fermentation products (Wilson 2005, Aminov *et al.* 2006). Besides being metabolically active, *E. rectale* plays a crucial role in the maintenance of overall gut homeostasis and participates in immune processes due to its habitat located adjacent to the immune cells in the intestinal mucosa (Atarashi *et al.* 2011, Nava *et al.* 2012). In addition, some of the cluster XIVa species such as *E. rectale* and *R. intestinalis* are motile since they possess flagella (FLG) (Neville *et al.* 2013) and thus harbor enormous immunostimulatory potential (Hayashi *et al*. 2001).

2.1.1.2 Actinobacteria: Bifidobacterium sp., Atopobium cluster

Actinobacteria are gram-positive, non-sporulating and non-motile obligatory anaerobes that include many important GM bacterial families such as *Bifidobacteriaceae* and *Coriobacteriaceae* (Ventura *et al.* 2007b). All *Actinobacteria* possess exceptionally high G + C content (46 to over 70 %) in their DNA. They are saccharolytic but do not produce gas as a result of fermentation (Wilson 2005). Traditionally, all members of the genus *Bifidobacterium* (to date almost 50 species distributed in six phylogenetic clusters) are nominated as probiotics, i.e., live microbes that endow health benefits to the host when administered in sufficient amounts (Ventura *et al.* 2007a, Bottacini *et*

al. 2014). *Bifidobacterium* are able to digest a large variety of polysaccharides such as dietary glycans (pectin), inulin and starch, the result of which is a somewhat acidic gut environment (Wilson 2005). This further inhibits the growth of other gut microbes and even enteropathogens such as *Salmonella.* Thus they are important players in the colonization resistance that protects the host from infections caused by the invasive pathogenic micro-organisms (Bottacini *et al.* 2014). Due to their various health promoting effects such as protection against the intestinal pathogens, immune modulation and most importantly the ability to attach to the intestinal epithelium or mucus layer concurrently preventing the contagion of pathogens, *Bifidobacterium* spp. are in active utilization as ingredients of so-called functional foods (Bottacini *et al.* 2014). In addition, some species are responsible for the production of the essential nutrients such as folic acid, vitamin K and B12 and degradation of bile acids (Ventura *et al.* 2007a, Ceapa *et al.* 2013). Dietary non-digestible, fermentable carbohydrates, i.e., prebiotics are called as nutritional "bifidogenic" factors since they stimulate the growth of these probiotic bacteria (Roberfroid *et al.* 2010). Thus prebiotics possess the ability to modify the GM structure. This feature has recently made the symbiotic therapeutic approach a popular way for the modification the GMC (Roberfroid *et al.* 2010, Bindels *et al.* 2014).

Atopobium cluster

To date quite little knowledge has been accumulated on the diversity and functionality of representatives of *Coriobacteriia,* namely the *Atopobium* cluster. The best-known representative of this cluster, *Collinsella aerofaciens,* has been recently acknowledged to be a member of a balanced GM (Qin *et al.* 2010, Gupta *et al* 2013, Thorasin *et al.* 2015). It seems that metagenomic approaches have under-represented *Atopobium* in many studies supposedly due to the relatively high G + C content in their DNA that exacerbates the amplification of DNA from this bacterium (Thorasin *et al.* 2015). For example, a recent study that evaluated the abundance and diversity of *Atopobium* from 13 human fecal samples by using both cultivation and molecular methods estimated that *Atopobium* represents 0.2 to 22 % of the total fecal bacteria, i.e., huge inter-individual variation was observed. However, it seemed that the *Atopobium* cluster was quite stable during the 3-month study (Thorasin *et al.* 2015). Recent human epidemiological studies have associated *Collinsella* genus with high serum total and low density lipoprotein (LDL) cholesterol (Lahti *et al.* 2013).

2.1.1.3 Bacteroidetes: Bacteroides spp. and Prevotella

Most abundant representatives of the gram-negative *Bacteroidetes* phylum include *Bacteroides, Parabacteroides, Alistipes, Prevotella* and *Porphyromonas* species that are non-sporing, non-motile rod-shaped anaerobes that maintain beneficial relationship with the host in the gut, but harbor high pathogenic potential if they reach the bloodstream as

a result of non-intact GI tract wall (Wexler 2007). For example *Bacteroides* spp. have been detected in various types of clinical specimens. Thus they can cause significant endogenous infections in multiple body sites (Wexler 2007). All genera produce succinic, acetic and propionic acid as a result of the fermentation process, and all except *Alistipes* are saccharolytic (Rajilic-Stojanovic & de Vos 2014)

Bacteroides spp. contribute significantly to the host-microbiota interactions, and to date nearly 30 species of *Bacteroides* have been isolated from human feces (Rajilic-Stojanovic & de Vos 2014). The most important representatives include *B. fragilis*, *B. dorei, B. ovatus, B*. *vulgatus* and *B. thetaiotaomicron* that all are quite easy to culture due to the rather simple nutritional requirements of the species (Wexler 2007). Frequent isolate extracted from the clinical specimens is opportunistic pathogen *B. fragilis,* which harbors various virulence factors in its genome (Wilson 2005). Enterotoxigenic *B. fragilis* strains (ETBFs) have been associated with the risk of colon cancer (Wu *et al.* 2009). In addition, recent associative studies have linked *Bacteroides* species, such as *B. dorei* and *B. vulgatus,* to the various inflammatory intestinal diseases (Sanchez *et al.* 2010, Sato *et al.* 2010, Bloom *et al.* 2011). In addition, *Bacteroides* seem to be enriched in both type 1 and type 2 diabetics' guts (Larsen *et al.* 2010, de Goffau *et al.* 2014).

The first *Bacteroides* spp. inhabit the infant's gut at a quite early phase during the colonization process, since breast milk is enriched with the nondigestible oligosaccharides that support the growth of *Bacteroides* (Marcobal *et al.* 2011). It seems that *Bacteroides* spp. colonize the GIT rapidly, efficiently and permanently since it has been shown that gut *Bacteroides* community is quite stable throughout the human life span (Rajilic-Stojanovic *et al.* 2012, Wrzosek *et al.* 2013). Intestinal *Bacteroides* spp. are very active in digestion, more specifically in the degradation of complex plant polysaccharides such as starch, pectin and cellulose based on the vast array of the respective enzymes encoded by their genomes (Xu *et al.* 2003).

B. thetaiotaomicron (Bttm) is a widely used model organism for the host-microbiota interactions. The whole Bttm genome has been sequenced, and it comprises 6.26 Mb and 4779 proteins including an abundant set of carbohydrate-degrading enzymes, CAZymes (Xu *et al.* 2003, Martens *et al.* 2008). In addition, the genome contains a variety of mobile elements such as transposons and plasmids, thus implying a capacity to horizontal gene transfer (Xu *et al.* 2003). CAZymes ferment otherwise indigestible dietary and mucus polysaccharides and acetate is produced as a result of this fermentation (Mahowald *et al.* 2009, Flint *et al.* 2012). If the nutritional reservoir of polysaccharides is scarce, Bttm may turn to the utilization of the mucus layer or vice versa (Martens *et al.* 2008).

Like *Bacteroides, Prevotella spp.* are also capable of the degradation of complex plant polysaccharides such as pectin and cellulose (Rajilic-Stojanovic & de Vos 2014).

Prevotella spp. colonize the oral cavity, and are quite often linked to oral diseases (Wilson 2005). For example *P.intermedia* is associated with periodontitis. However *Prevotella* spp. appear also to be notable members of GM and have been associated with many clinical conditions (Zhang *et al.* 2009). *Prevotella* spp. ferment various carbohydrates such as cellulose and xylan producing acetate and succinate (Wilson 2005).

2.1.1.4 γ-Proteobacteria: family Enterobacteriaceae

The versatile and well-defined family of the gram-negative *Enterobacteriaceae* (enterobacteria) belongs to a healthy human GM. However, their abundance is quite low – less than 1 % of total microbiota in the large intestine (Holdeman *et al.* 1976, Hopkins *et al.* 2001, Wilson, 2005, Rajilic-Stojanovic & de Vos 2014). Enterobacteria comprise of at least 30 genera and 150 species that are gram-negative, non-sporing, facultatively anaerobic rods that ferment glucose and produce acetic, succinic and lactic acids as well as CO_2 and H_2 as end products (Wilson 2005). Enterobacteria are commonly associated with acute GI infections and diarrhea (Thielman & Guerrant, 2004). However, most are opportunists and thereby are infectious only for example through the surgical wounds or when the gut barrier is ruptured (Wilson 2005).

The first isolated and the most prevalent representative of enterobacteria is the well-studied *Escherichia coli*, which colonizes the mammalian GIT already a few hours after the birth (Favier *et al.* 2002). *E. coli* is a widely used model organism in biochemical and molecular microbiology laboratories. It is known especially for the ability to cause GI and urinary tract infections (Wilson 2005). Pathogenic strains harbor a set of virulence genes (so-called pathogenicity island) that code for example toxin production or invasiveness and are absent from the gut commensals (Wilson 2005). However, some strains of *E. coli* are even used as probiotics (Grozdanov *et al.* 2004). Other important genera that are found from GI tract are *Citrobacter*, *Enterobacter*, *Hafnia* and *Salmonella* and *Serratia* (Wilson 2005, Rajilic-Stojanovic & de Vos 2014).

Probably the most important virulence factor that Enterobacteria possess is outer membrane lipopolysaccharide (LPS). The toxicity potential of the LPS has been recognized already over 100 years ago. Since residing in the outer membrane, i.e., within the bacteria, LPS belongs to endotoxins and it consists of three structural parts: lipid A that is embedded in the outer membrane and is actually responsible for the toxic effects, surface-situated core polysaccharides and O-antigen repeats (reviewed in Raetz & Whitfield 2003). LPS structures and consequently virulence efficiency varies between the different gram-negatives. The host's innate immune system defends against the gram-negative bacteria by utilizing specific recognition molecule called Toll-like receptor 4 (TLR4) (Medzhitov *et al.* 1997). In addition, most members of this family contain flagella that enable their movement

2.1.1.5 Akkermansia muciniphila

The anaerobic phulum *Verrucomicrobia* consist mainly of gram-negative *Akkermansia muciniphila* species that has been linked to the maintenance of good gut health (Derrien *et al.* 2004, Png *et al.* 2010, Derrien *et al.* 2008). *A. muciniphila* genome was published recently revealing that this bacterium has a specific niche in GM (van Passel *et al.* 2011). For example *Akkermansia* is specialized in living in the mucin layer near mucosa and is efficient utilizer of mucin *i.e.* glycosylated proteins that coat and protect the intestinal epithelium, and is capable to survive even if mucin serves as its sole carbon source (Derrien *et al.* 2004, van Passel *et al.* 2011). The relative abundance of *Akkermansia* varies reaching over 3% of the total bacterial count in adults (Derrien *et al.* 2008).

Studies in mice have suggested that *A.muciniphila* has a causal role in the improvement of host's health. Oral administration of this bacterium has been associated with increased mucin layer thickness and improved inflammatory status in mice (Everard *et al.* 2013). In addition, the greater fecal abundance of *A.muciniphila* has been linked to the normal body weight, lower body mass distribution and better glucose tolerance in mice as well as normal glucose tolerance in humans (Everard *et al.* 2013, Zhang *et al.* 2013, Joyce & Gahan 2014). However, also the controversial results regarding the role of *A.muciniphila* in metabolic health in humans have been suggested since its enrichment was recently associated with T2D (Qin *et al.* 2012).

2.1.1.6 Non-bacterial players

Recent metagenomic studies have revealed that even healthy individuals harbor viruses, eukaryotes and *Archaea* side by side with bacteria in the gut (Eckburg *et al.* 2005, Rajilic-Stojanovic & de Vos 2014). It seems that there is an enormous viral community in the GIT all of which together form a virome. Most of the detected viral-like particles (VLP) are actually bacteriophages, i.e., viruses that infect bacteria. In addition, the population constitutes viruses that infect both the host cells and organisms of eukaryome (Minot *et al.* 2011, Virgin 2014). Like in the bacterial population, the huge inter-individual variability characterizes also viromes and eukaryomes. However, both are relatively stable over time as for example over 95 % of the individual VLP sequences remained stable during the time period of one year (Reyes *et al.* 2010). In addition, no signs of higher similarity in genetically-related subjects versus non-relative ones were detected. The deep sequencing approaches have revealed a set of novel VLP. Thus comprehensive studies are still needed in the future to further reveal the whole diversity (Virgin 2014).

Archaea, mainly the genus *Methanobrevibacter,* have been isolated from the human feces and their presence in human gut has been confirmed by the sequencing approaches (Dridi *et al.* 2009). *Archaea* are considered as a distinct domain – its representatives are neither bacteria nor eukaryotes. The existence and prevalence of *Archaea* in the GI tract varies a lot between individuals (Eckburg *et al.* 2005).

Gut also harbors eukaryotic microbes that are considered as symbionts instead of the interpretation of being viewed as parasites. However, the degree of species and diversity is currently not well known, though Next Generation Sequencing (NGS) approaches have recently provided much more knowledge about the eukaryotome (Parfrey *et al* 2014). Yeasts, protists (*Blastocystis*, Amoeboids) and fungi were frequently detected by NGS from the fecal samples of healthy westerners and Africans. However, distinct inter-individual eukaryotome differences were observed, suggesting that these are driven by the differences in the dietary patterns. For example Africans seem to have a much higher eukaryotic biomass than westerners (Parfrey *et al* 2014).

2.1.2 Development and stability of gut microbiota

Traditional consensus assumes that mammalian fetuses are kept in sterile conditions in uterus and thus safe from the infections. Interestingly, the presence of bacteria in 48 placentas was recently reported and further identification of the species by metagenomic sequencing revealed that this bacterial community mostly resembled the one in the mouth (Aagard *et al.* 2014). Therefore it is possible that the first bacterial exposures occur already in the womb during the pregnancy.

Microbes start to colonize newborn's gut and other body compartments during the birth and immediately after it (Palmer *et al.* 2007). Although first fecal sample, meconium, is considered to contain a very low amount of microbes, the colonization proceeds rapidly (Koenig *et al.* 2011). The sort of first colonizers and progression of the process depend primarily on the mode of delivery, since the GMs of vaginally born infants differ significantly from the ones delivered by caesarian (C-) section (Palmer *et al.* 2007, Dominguez-Bello *et al.* 2010). Further on feeding (breastfeeding vs. formula), possible early exposure to antibiotics and the stage of sanitation have major impact on the maturation of microbiota at early age. In addition, host genotype and physiological gut development have an impact on GMC (Palmer *et al.* 2007, Collado *et al.* 2015). As early infancy is also a critical time period for the maturation of the immune system (IS), the simultaneously occuring GM assembly and diversification are utmost important processes in terms of health (Maynard *et al.* 2012).

During the first few years of life GMC is at constant fluctuation (Ottman *et al.* 2012). The GM of solely and mainly breastfed babies is dominated by the *Bifidobacterium* species, and differ from their formula-fed counterparts that have more diverse and "adult-type" GM already at this time (Goldsmith *et al.* 2015). In the Western world the first solid foods are usually introduced at the age of four months, and the nutritional choices made have a great impact on the further GM diversification process. During this time, the dominance of *Bifidobacteria* is ceding, and *Bacteroides* sp. and various *Clostridium* clusters take over space (Verdu *et al.* 2015). Adult-type, more or less stable structure is established

roughly by the age of two to three years (Palmer *et al.* 2007, Koenig *et al.* 2011, Ottman *et al.* 2012, Yatsunenko *et al.* 2012). During the whole adulthood, GM is considered to remain relatively constant and is characterized by the so-called stable colonizer species. However, slight composition evolvement occurs during the whole life span and GM constantly adapts to various external stimulus (Rajilic-Stojanovic *et al.* 2012, Ottman *et al.* 2012). Some age-related destabilization occurs again in the senescence and notable inter-individual variation exists also during the aging process (Claesson *et al* 2011, Biagi *et al.* 2012, Claesson *et al* 2012, Rampelli *et al.* 2013). A recent systematic review summarized the features of elderly subjects' GM profile. It expresses reduced overall diversity and at phylum level contains less *Firmicutes* and higher presence of *Bacteroidetes* than the adult GM. Lower amount of *Bifidobacterium, F. pravsnitzii* and some members of *Clostridium* cluster XIVa are also associated with older age (Rondanelli *et al.* 2015). Decreased amounts of obligatory anaerobes and significant increase of facultative anaerobic members such as *Streptococcus, Enterococcus* and Enterobacteria in elderly compared to their younger peers have been reported (Biagi *et al.* 2012).

Worldwide, human milk is considered to be the best nutritional choice for the first months of the infant's life since it includes an optimal ensemble of factors that have health-promoting potential (Walker 2010). In addition to carbohydrates, nucleotides, fatty acids and immunomodulatory factors, breastmilk also contains bacteria, i.e., harbors a microbiome of its own (Walker 2010, Cabrera-Rubio *et al.* 2012).

2.1.3 Healthy gut microbiota, core microbiome and enterotypes

GM is an ecosystem with exceptional plasticity and a high degree of inter-individual structural variability that describes both healthy and diseased human communities (Eckburg *et al.* 2005, Gill *et al.* 2006, Turnbaugh *et al.* 2009a, Robinson & Young 2010). Intra-individual differences are recognized both between different body habitats and over time and even within site, thus it has been extremely challenging to build up the unambiguous definition for the healthy, balanced GM structure. Obviously, the lack of such a definition complicates the identification of diseased GMC. Therefore such a definition is a prerequisite for the recognition of diseased GMC (Costello *et al.* 2009, Clemente *et al.* 2012, Lahti *et al.* 2014). Although there still is a lack of an unambiguous definition, many recent studies have identified certain bacterial species and ecological features that could serve as markers for healthy microbiota (Walker and Lawley 2013).

The term dysbiosis, which refers to an aberrant GMC that causes distubances in the intestinal homeostasis, has been recently proposed (Burcelin 2012, Walker and Lawley 2013, Carding *et al.* 2015). GM signatures related to the dysbiosis include reduced overall bacterial diversity or low abundance of health promoting members of GM with respect to potentially harmful (e.g. pro-inflammatory) bacterial species (Sekirov *et al.* 2010,

Burcelin 2012). Dysbiosis can be caused by various factors that influence the GMC, such as unfavorable lifestyle habits and genetic vulnerability (Burcelin 2012). It has been recently associated with the various inflammatory diseases and disorders such as allergy, inflammatory intestinal diseases and obesity, since it seems to impair the integrity of the gut barrier, which leads to the translocation of inflammatory gut microbes and their components into the bloodstream that further may cause systemic low-grade inflammation (SLGI, reviewed in Sekirov *et al.* 2010, Clemente *et al.* 2012). Deviations from the overall balance quite often result from the temporary enteric pathogen overgrowth, dietary factors or repeated use of antibiotics (Vaahtovuo *et al.* 2007, Robinson & Young 2010).

One of the main goals of different international microbiome programs such as the MetaHit consortium has been the constitution of a definition of the so-called core microbiota or microbiome, i.e., a definition of the set of conserved organisms, genes and functions that are shared among all or vast majority of healthy individuals (Tap *et al.* 2009, Sekirov *et al.* 2010). Even though enormous inter-individual variability in GM exists, some bacterial species seem to be conserved between most of the people - thus the existence of a phylogenetical core of only dozens of microbiota species has been suggested (Turnbaugh *et al.* 2009, Costello *et al.* 2009). The numerical abundance of certain GM members does not necessary equate to their functional relevance. The 66 operational taxonomic units (OTUs) determined from 17 individuals by high throughput pyrosequencing approach corresponded to 38 % of all the sequence reads (Tap *et al.* 2009).

Few years ago MetaHit introduced the concept of enterotypes that point to the co-existence of some bacterial genera and species in human feces (Arumugam *et al.* 2011). Subjects had been divided into three groups based on their distinct fecal GM patterns, i.e., enterotypes (Arumugam *et al.* 2011, Koren *et al.* 2013). Enterotype (ET) 1 is dominated by *Bacteroides* spp, ET 2 by *Prevotella* spp., and gram-positive *Ruminococcus* spp. is enriched in ET 3. Six different nationalities were included in the study population (n = 39), and compositional differences were detected independently of age, gender, body mass index (BMI) and nationality. Similar compartmentalization of the GIT bacterial species was later detected in chimpanzees, with a correspondence between those and human enterotypes. Thus it is possible that the enterotypic variation in humans has an ancestral feature (Moeller *et al.* 2012).

These findings have created an interesting new outlook into the development of microbiota-related diagnostics and therapies since the classification of individuals to a limited number of categories based on their gut contents seems to be a highly tempting approach. In addition, the three enterotypes were further analyzed with respect to diet, and a clear link between long-term dietary habits/behavior and two different enterotypes was found: ET1 type associated with high fat or protein diets and ET2 type with high carbohydrate diet (Wu *et al.* 2011). However, further studies utilizing European (n=663)

and US population (n = 12) revealed that the borders between the enterotypes are not exactly clear and that some bacterial groups and genera frequently appear together as co-abundance groups (Koren *et al.* 2013). Instead of the enterotype classification, a growing body of evidence supports the co-occurrence and co-exclusion of the various GM species (Faust *et al.* 2012). Thus, it seems that GM maybe should be viewed more as gradients or continuum than as discrete bacterial communities and that the original definition of enterotypes may oversimplify the matter (Jeffery *et al.* 2012).

2.1.4 Methods to study gut microbiota composition

Traditionally gut bacteria have been analyzed by culture, which basically means the ability to grow viable bacteria *in vitro,* i.e., outside their natural habitat such as gut (Rajilic-Stojanovic & de Vos 2014). However, major significant gut bacteria especially in colon are strict anaerobes and require specific, complex culture media for growth (Rajilic-Stojanovic & de Vos 2014). Currently it is commonly estimated that as much as 80% of the bacterial species found by molecular tools from the human gut are either uncultured or even impossible to culture (Turnbaugh *et al.* 2007, Rajilic-Stojanovic & de Vos 2014). However, estimations vary a lot depending on the source or reference (Rajilic-Stojanovic & de Vos 2014). Recently, various modern molecular techniques have been applied for the analysis of the entire bacterial communities, which enables the analysis of both cultivated and non-cultivated members (Goodrich *et al.* 2014a). Figure 2 summarizes currently mostly used DNA-based molecular analysis methods. This literature review introduces mainly targeted phylogenetic analysis approaches.

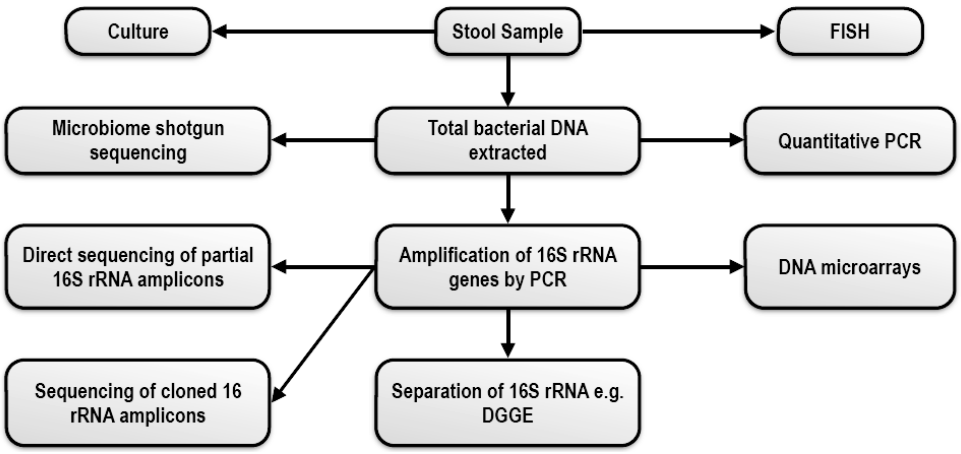

Figure 2. A summary of molecular microbiology methodologies currently utilized in gut microbiota research. DGGE = denaturing gradient gel electrophoresis, FISH = fluorescense *in situ* hybridization, PCR = polymerase chain reaction

The culturable genera of gut bacteria include for example *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium* and *Ruminococci* (Wilson 2005, Rajilic-Stojanovic & de Vos 2014). In addition, less abundant but important bacteria such as facultative anaerobic Enterobacteria, *Lactobacilli* and *Streptococci* are easy to isolate from gut matter and culture *in vitro* (Wilson 2005). Culture still has its advantages since the isolation and culture of bacteria from biological samples is the only way to perform physiological characterization and to get fast information regarding the metabolism and nutritional and conditional growth requirements (Rajilic-Stojanovic & de Vos 2014). However, recent explosion in the usage of NGS and other molecular microbiology methods has led to the delinquency of culture in microbiology laboratories (Lagier *et al.* 2012). However, for the successful understanding of GM, knowledge on both the isolation and culture of bacteria is still urgently needed. Thus, there has been a renewed interest in culture methods for 'non-cultivable' species (Lagier *et al.* 2012).

Recently, the concept of culturomics was introduced by a French study group (Lagier *et al.* 2012). It describes the modern, high-throughput culture-approach that is more or less analogous to the metagenomics approach in aiming for unravel the GMC (Lagier *et al.* 2012). Culturomics combines the utilization of a large set of selective and enrichment culture media and conditions with the step of identification of bacterial species, by MALTI-TOF mass spectrometry. Over 210 different culture conditions have been applied yielding 32,500 colonies which represented 340 bacterial species, five fungi and several viruses. Importantly, representatives from two rare phyla (*Deinococcus-Thermus* and *Synergistetes*), and altogether 174 species that had not been described previously were found from the fecal samples of three individuals (Lagier *et al.* 2012).

2.1.4.1 Sampling and storage

Sample material in human GM studies is feces, rectal swabs or endoscopic biopsies from the colon or small intestine. The sampling sets major challenge to the analysis process since human feces is an extremely complex ecosystem containing a great variety of different microbial groups and species (Zoetendal *et al.* 2004, Vandeputte *et al.* 2015). Extra challenges arise from the fact that GIT and fecal samples also contain abundant amounts of material of non-microbial origin such as fibers from food and this debris material may disrupt the successful performance of many downstream methodologies (Zoetendal *et al.* 2004, Vaahtovuo *et al.* 2005,Vandeputte *et al.* 2015).

The pre-requirement for the sampling procedures and storage conditions is that they should not alter the quality and most importantly the microbial structure of the samples (Salonen *et al.* 2010, Maukonen *et al.* 2012). Rapid freezing and storage of the specimens in – 80 °C is the most employed way of sample storage in large human cohorts due

to practical reasons, since it is usually impossible to perform downstream processing immediately after the sampling (Bahl *et al.* 2012). Alternatively, various types of sample preservatives such as alcohol fixatives or commercial products such as RNA*later* by Qiagen have been exploited. However, many studies have shown that even freezing and preservatives may affect the microbial community structure (Salonen *et al.* 2010, Maukonen *et al.* 2012, Bahl *et al.* 2012, Fouhy *et al*. 2015).

Long-term storage in the deep-freezing conditions may alter the GMC (Rochet *et al.* 2004). In addition, the exposure of the GI tract samples to room temperature for different time periods prior to freezing has an influence on their microbial composition (Carroll *et al.* 2012). Still great controversy remains on whether the samples must be immediately frozen or whether they can tolerate room temperature for a period of time. In conclusion, whatever the conditions used, it is most important to be consistent and perform actions in the same manner across all the samples of the study (Vaahtovuo *et al.* 2005, Goodrich *et al.* 2014a).

Recently, various important GM research groups and laboratories have together founded The International Human Microbiome Standards (IHMS) project. The aim of the project was to coordinately create unified and standardized operating procedures (SOPs) for the microbiome research field (http://www.microbiome-standards.org/). Currently the focus has been on the beginning of workflow in sampling and storage of samples and in DNA extraction.

2.1.4.2 Molecular methods based on the sequence variability of 16S rRNA gene

The universal key marker gene utilized in various GM analysis applications codes for the small subunit of 16S ribosomal RNA (16S rRNA) that has been for decades considered as a measure of the evolutionary proximity of organisms (Woese & Fox 1977). The 16S rRNA gene is highly conserved among bacteria and has remained almost unchanged during the evolution. In addition to the highly conserved areas, nine regions of variable nucleotides exist within the 1, 500 bp gene and these nine regions constitute enough information for the use of successful taxonomic classification since its sequence depicts evolutionary distances (Woese & Fox 1977). Nine hypervariable regions within the gene can be used in the designing of targeted oligonucleotide probes and primers, and bacterial species level can be detected (Amann 1995).

Fluorescense *in situ* hybridization (FISH)

Applicable, quantitative phylogenetically targeted method for the identification and quantification of the different bacterial groups and genera from a mixed bacterial sample is fluorescense *in situ* hybridization (FISH), i.e*.,* detection of the fixed, whole bacterial

cells with fluorescent 16S rRNA-targeting oligonucleotide probes (Rochet *et al.* 2004, Vaahtovuo *et al.* 2005). The FISH technique is not dependent on the extraction and amplification of the bacterial DNA that could cause bias to the analysis results (Zoetendal *et al.* 2004, Vaahtovuo *et al.* 2005).

The hybridization step in the FISH is usually combined either with microscopy or a cell counter such as flow cytometry (FCM). Briefly, this technique utilizes short sequence-specific probes that attach to the complementary nucleic acid sequence of the bacterial cells of interest (Amann *et al.* 1995). One bacterial cell may contain hundreds or even thousands of 16S rRNA molecules. The detection in FISH is based on fluorochrome that is adhered to the probe. Attachment occurs into the compatible 16S rRNA structural units of the ribosomes. Phylogenetic targeting enables the accurate detection of certain bacterial groups or even species, depending on the specificity of the probes (Amann *et al.* 1995, Wagner *et al.* 2003, Vaahtovuo *et al.* 2005). However, the development of FCM-FISH analysis for bacteria has been quite a challenge due the fact that bacteria are considerably smaller than for example human cells (Zoetendal *et al.* 2004). In addition, common fluorochromes such as fluorescein do not emit sufficiently intensive light for the detection of bacteria in FCM (Rigottier-Gois *et al.* 2003, Zoetendal *et al.* 2004,Vaahtovuo *et al.* 2005). The challenges of the FCM-FISH technology are sometimes detected at low signal intensities due to impermeability of the fixed bacteria and a low cellular rRNA content. In addition, the three-dimensional structure of the ribosome may hinder the access of oligonucleotides to their target sites (Wagner *et al.* 2003). Traditionally the FCM-FISH methodology utilizes the general probe, Eub338, which should theoretically bind to the highly conserved region (338-355 *E. coli* numbering) of the 16S rRNA molecule of all the bacteria in a sample (Rigottier-Gois *et al.* 2003, Amann *et al.* 1990). However, there are studies reporting that Eub338 does not detect all the bacteria in a mixed sample (Daims *et al.* 1999, Bouvier & De Giorgio 2003, Vaahtovuo *et al.* 2005). In order to outrival the frailty of the Eub338 probe, an analysis based on general DNA-staining has been developed (Zoetendal *et al.* 2004, Vaahtovuo *et al.* 2005). By using the DNA-staining combined with the FCM-FISH it is possible to distinguish the hybridized and DNA-stained target bacteria from the rest of the bacteria and debris more efficiently as represented in Figure 3.

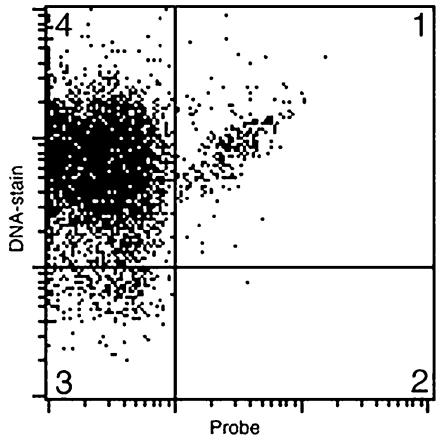

Figure 3. An example of the FCM-FISH analysis of the human fecal sample. The target bacteria of interest are shown in region 1 and all the other bacteria in region 4. The DNA-stain negative and probe negative particles reside in region 3 and false-positives in region 2. (Vaahtovuo *et al.* 2007).

Next Generation Sequencing (NGS)

It is meaningful to analyze whole microbial ecosystems and not their predefinated, single representatives since microbial metabolic processes always occur in communities in nature (Qin *et al.* 2010). Nowadays, the most popular way for targeted phylogenetic analysis seems to be NGS approaches such as MiSeq (Illumina) and 454 (Roche) that give an estimation of the bacterial and archaeal species richness based on all the 16S rRNA sequences in a given sample (Goodrich *et al.* 2014a). As a result, question "Who is there?" can be answered (Goodrich *et al.* 2014a). Briefly: extracted DNA is subjected to the PCR amplification using primers designed to attach to the variable regions in 16S rRNA gene. Gained amplicons are further sequenced by some NGS platform such as MiSeq. After the quality-filtering steps each sequence from the sample is then further assigned to taxonomical classification performed by phylogenetic analysis softwares (Goodrich *et al.* 2014a, Wieland *et al.* 2015).

NGS produces an enormous amount of data, and it has been quite a challenge to set up the bioinformatics pipelines and pick up correct data handling tools. Currently, the most widely used example is QIIME, Quantitative Insights Into Microbial Ecology software package (Caporaso *et al.* 2010). Besides sequencing, 16S rRNA gene-based microarrays have been developed for the community-wide, fingerprinting analysis of GMC. One example of such molecular arrays is the human intestinal tract chip (HITChip) (Rajilic-Stojanovic *et al*. 2009).

First studies aiming for the microbiome analysis were performed by traditional Sanger method and sequenced clone librariers from the human fecal samples (Gill *et al*. 2006). However recent rapid development in sequencing strategies has enabled the development of metagenomics, i.e., the non-targeted analysis of all the microbial genes within a sample

(Morgan & Huttenhower 2014, Goodrich *et al.* 2014a). As a result, both diversity and the whole genetic potential of a given sample is solved (Goodrich *et al.* 2014a) and also non-bacterial members such as viruses are detected since metagenomics is performed by the shotgun or high-throughput parallel sequencing (for example HiSeq by Illumina) of microbial DNA (Morgan & Huttenhower 2014, Goodrich *et al.* 2014a) Current advanced status of the metagenomics techniques has enabled the build up of extensive gene catalogues from the samples of interest (Qin *et al.* 2010, Karlsson *et al.* 2013, Li *et al*. 2014).

2.2 Factors that determine and shape the composition and function of GM

Numerous factors influence on the structure, diversity and species richness of GM (Figure 4). Both host-related factors such as age, gender, genetics and diseases and environmental determinants such as diet, lifestyle, geographical location and hygiene have been recognized (reviewed in Sekirov *et al.* 2010, Spor *et al.* 2011, Candela *et al.* 2012, Salonen 2013). However, the extent of the effects of various above-mentioned factors still remains under investigation. Understanding the role of the individual-specific factors is a necessity for the intentional GM manipulation for therapeutic purposes as a tool of personalized medicine in the future.

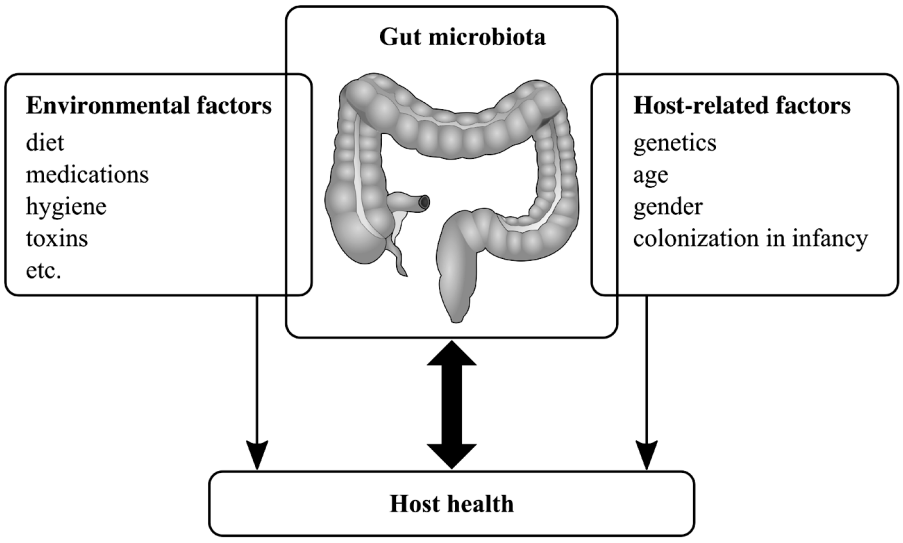

Figure 4. Factors affecting gut microbiota composition. Modified from (Salonen 2013).

GMC and the host-microbe interactions are affected by the genetics of the hosts (Vaahtovuo *et al.* 2003, O'Connor *et al.* 2014). It seems that the individuals that are genetically related to one another have more similar GMCs than non-relatives, but this factor may also arise from the shared environment and dietary habits (Turnbaugh *et*

al. 2009, Vaishampayan *et al.* 2010). Thus, the environmental factors are likely to rule over the effect of genetics and are thought to be the key determinants of GM structure (Zhang *et al.* 2010). For example, in *Apoa*-I knockout mouse model that is predisposed to obesity and MetS dietary changes explained nearly 60 % of GM variation observed while genetics explained only 12 % (Zhang *et al.* 2010). In addition, various studies have proposed that no significant difference in GM structure exists between monozygotic and dizygotic twins (Turnbaugh *et al.* 2009a, Tims *et al* 2013, Goodrich *et al.* 2014b, Lim *et al.* 2014, Murphy *et al.* 2015). For example the study conducted with 977 twins pairs found no significant differences in overall GMC between mono- and dizygotic twins (Goodrich *et al.* 2014b). However, the heritability of certain gut bacteria was more similar in identical than in non-identical twins and the most heritable bacteria belonged to the *Christensenella* genus. Intriguingly, the groups of bacteria that were heritable are the ones that have previously been linked to diseases suggesting novel mechanisms for the genetic inheritance of diseases (Goodrich *et al.* 2014b).

Specific host genes are recognized for their ability to shape the GM structure. One of the most interesting candidates that play an important role in the determination of GMC during colonization is the gene that encodes bacterial flagellin-recognizing Toll-like receptor 5 (TLR5) (Leifer *et al.* 2014). Single nucleotide polymorphisms (SNPs) in *TLR5* may affect the functionality of the receptor and have been further linked to the inflammatory status of the gut and also to various other diseases (Leifer *et al.* 2014). Interestingly, specific genetic factors that are predisposing to autoimmune or lifestyle diseases, such as *FUT2* may also influence GMC, and this may further promote the incidence of diseases (Spor *et al.* 2011, Rausch *et al.* 2011, Marietta *et al.* 2015). In addition, it is known the GM colonization process in early childhood is a genetically regulated event. However clear evidence on to what extent the overall host genetics matter still remain scarce in humans (Ley *et al.* 2006, Goodrich *et al.* 2014b).

2.2.1 Gender

Gender-based differences of GM have been reported in mice. These differences seem to appear only after animals reach the sexual maturity (Yurkovetskiy *et al.* 2013, Markle *et al.* 2013). Sex hormones seem to be the key players also in the regulation of the genes related to the adaptive and innate immunity and inflammation (Yurkovetskiy *et al.* 2013). To date the data regarding the effect of gender on GM has been inconclusive in humans although some gender-related signatures have been found (Mueller *et al.* 2006, Dominianni *et al.* 2015). For example a recent study (n=82) showed that women had significantly lower fecal abundance of the members of the *Bacteroidetes* phylum compared to men (Dominianni *et al.* 2015). Interestingly, low levels of the gram-negative *Bacteroidetes* have been associated with female gender also in mice. Thus hormonal

mechanisms and especially sex steroid levels seem to have an impact on the microbiota (Yurkovetskiy *et al.* 2013).

For example, incidences and/or severity of autoimmune diseases such as juvenile diabetes are more prevalent in females (Markle *et al* 2014). Thus, there are substantial gender-based differences with respect to basic physiology, body composition, immune responses and susceptibility to a broad variety of diseases (Markle *et al.* 2014). Evidence that the early microbial exposure affects sex hormone levels have been presented by using the particular NOD mouse model for T1D (Markle *et al.* 2013). The incidence of T1D decreased in the female NOD mice as testosterone expression was induced by the indigenous GM. Further, transplantation of the male gut content into the immature female recipients resulted in the increased testosterone production, therefore supporting the existence of sex hormone-microbiome relationship (Markle *et al.* 2013).

2.2.2 Diet and gut microbiota

Diet is probably the most important singular factor that shapes the GMC since food consumed by the host simultaneously feeds the hundreds of trillions of bacteria in the GI tract (Wu *et al.* 2011, Scott e*t al.* 2013, Salonen & de Vos 2014, Sonnenburg & Sonnenburg 2014). For example, in a study conducted with humans and 59 other mammalian species, the gut metagenomes of carnivores, omnivores and herbivores each clustered separately (Ley *et al.* 2008). In addition, diet-microbiota interactions are likely to play a crucial role in the link between the GM and health since different components of the diet are known to activate different bacterial genes in the microbiome (Kau *et al.* 2011, Wu *et al.* 2011). It is estimated that the diet outweighs the effect of the genetics on GM (Ley *et al.* 2008, Zhang *et al.* 2010, Muegge *et al.* 2011). Mueller *et al.* studied already almost a decade ago country-related differences in the fecal microbiota composition. The level of *F. prausnitzii* was highest in the Swedish study group compared to the Italian, German and French groups. The difference was explained by the dietary habits (Mueller *et al.* 2006). Recent, well-controlled human intervention (n=98) evaluated the association of the so-called GM enterotypes with the specific diets (Wu *et al.* 2011). *Bacteroidetes* ET seem to be positively associated with the long-term consumption of high animal-fat/protein diet, and *Prevotella* on the other hand with the consumption of carbohydrates. Further, ten individuals participated in a ten-day controlled feeding study, where the effects of high fat/low fiber (HF/LF) and low fat/high fiber diets (LF/HF) on the GMC were compared.

From the numerous dietary components that modify the GMC, fiber is known for its various beneficial health effects such as the reduction of the gut transit time and binding of fecal carcinogens both of which protect from colon cancer (Roberfroid *et al.* 2010). Fibers and other complex, dietary carbohydrates are indigestible in the upper parts of the

GI tract and metabolized in the colon by the gut bacteria to SCFA that further affect the host metabolism and health (Cummings & MacFarlane, 1997, Sonnenburg & Sonnenburg 2014). Fermentation is possible since the colon microbiome possess genes for the various carbohydrate-active enzymes (Turnbaugh *et al.* 2006, El Kaoutari *et al.* 2013).

SCFA are probably the most important metabolites that are produced in the colon via GM fermentation process (Cummings & MacFarlane, 1997, Sonnenburg & Sonnenburg 2014). The most important SCFA are acetate, propionate and butyrate that are produced in the ratio of 60:25:15 (Macfarlane & Macfarlane 2003). They all are rapidly absorbed in the intestine (Cummings & MacFarlane 1997, Hartstra *et al.* 2015). Butyrate is one of the most studied health-promoting metabolites. A lack of this metabolite in the gut has been associated with various inflammatory intestinal diseases (Hamer *et al.* 2008, Sokol *et al.* 2008, Sokol *et al.* 2009). Members of the gram-positive *Clostridium* clusters IV or XIV are the main produces of butyrate that further has various functions such as serving as an energy source for the colonic epithelial cells, improvement of the integrity of the gut barrier and alleviation of the inflammation (Hamer *et al.* 2008, Sokol *et al.* 2008). For example, experimental oral sodium butyrate treatment has been shown to cause systemic anti-inflammatory effect both in Crohn's disease and ulcerative colitis patients (Vernia *et al.* 2000, Di Sabatino *et al.* 2005).

The remaining two SCFA, acetate and propionate, enter the bloodstream via the portal vein after which acetate is metabolized in muscles or serves as a precursor for cholesterol synthesis and propionate in liver affecting hepatic *de novo* lipogenesis and gluconeogenesis (Cummings & MacFarlane 1997, Hartstra *et al.* 2015). Thus SCFA participate in the regulation and control of various metabolic pathways in humans (Cummings & MacFarlane 1997, Salonen & de Vos 2014, Sonnenburg & Sonnenburg 2014, Hartstra *et al.* 2015).

2.2.2.1 Western diet and GM

The diversity and richness of GM seems to be decreased due to the long-term consumption of typical Western food that includes too high amounts of fat (high-fat diet, HFD) and low amounts of complex carbohydrates (reviewed in Salonen & de Vos, 2014, Sonnenburg & Sonnenburg 2014). HFD does not provide the nutritional elements required for balanced GM function since most of the foodstuffs are absorbed already in the upper parts of the digestion system without reaching the lower parts of the small intestine and colon (Bengmark 2013, Sonnenburg & Sonnenburg 2014). For example, the GM structure of Italian children (n=15) had significantly reduced diversity compared to their healthy counterparts of the same age in Burkina Faso (n=14). The diet resembling early humans and rural living conditions and in Africa were thought to be the explanation for the detected differences (De Filippo *et al.* 2010). In addition, HFD seems to play a role in

the induction of insulin resistance (IR) and glucose intolerance (Cani *et al.* 2007a). It has been also reported to promote the buildup of obesogenic GMC (Bäckhed *et al.* 2007, Cani *et al.* 2007b, Cani *et al.* 2008). Interestingly, GF mice consuming HFD seemed to resist the excess weight gain and further ramifications such as inflammation in the adipose tissue and IR (Bäckhed *et al.* 2007, Rabot *et al.* 2010).

The type of the dietary fat seems to have a great impact on the amplitude and features of the interaction between GM and metabolic disorders (Caesar *et al.* 2015, Cani *et al.* 2008). In human studies, polyunsaturated fatty acids (PUFA) have been associated with the lean and metabolically healthy phenotype and their addition to the diet seem to alleviate inflammation in white adipose tissue (Calder *et al.* 2006, Buckley & Howe 2010). Recently in an 11-week dietary intervention it was shown that the mice fed with saturated fat (lard) expressed a different phenotype and GMC than mice fed with fish oil (Caesar *et al.* 2015). Significantly lower phylogenetic diversity was observed in the lard group compared to the fish oil group that harbored significantly more *Akkermansia muciniphila, Bifidobacterium* and *Lactobacillus* in their cecum. In the fish oil group increased insulin sensitivity and a decreased level of adipose tissue inflammation were detected during the intervention. On the other hand the lard-fed mice harbored more gram-negative *Bacteroides* and specific pathobiont *Bilophila wadsworthia* and had the increased fasting glucose and insulin levels and TLR activation. However, cecal SCFA levels were similar in the two groups (Caesar *et al.* 2015). Milk fat induced the abnormal growth of *B. wadsworthia* in the IL10-deficient mice (Devkota *et al.* 2012). Simultaneously mice suffered from the colitis triggerd by the increased taurolic acid production caused by colitogenic *B. wadsworthia* (Devkota *et al.* 2012).

2.2.3 The effect of antibiotics on GM composition

The consumption of antibiotics usually induces disturbances in the GMC (Dethlefsen *et al.* 2008, Schulfer & Blaser 2015). The restriction of the diversity and species richness are common consequences (Sullivan *et al.* 2001, Dethlefsen *et al.* 2008, Jernberg *et al.* 2010). However, it is challenging to draw any common conclusions about the effects, since like in the studies related to diet (Korpela *et al*. 2014, Salonen *et al*. 2014), the response of each individual seems to be unique. Then again it is also quite impossible to predict how the loss of certain bacteria affects a certain individual (Dethlefsen & Relman 2011). The over usage of antibiotics has also boosted the development of the antibiotic-resistant pathogens (Dethlefsen *et al.* 2008).

The observed change and recovery of the GMC strongly depends on the treatment, i.e., the duration, dosage and type of antimicrobial that has been used (Schulfer & Blaser 2015). For example, one-week treatment with clindamycin significantly reduced diversity of the *Bacteroides* and the effect remained distinctive even two years after the

course (Jernberg *et al.* 2010). On the other hand, the oral five-day course of ciprofloxacin led to the reduction of the gram-positive *Clostridiales* and changed the overall GMC in three healthy humans by 30 %. The microbiota recovered from this treatment to the pre-antibiotics level within four weeks after the end of the exposure (Dethlefsen *et al.* 2008). However, when ciprofloxacin treatment was repeated twice, highly variable responses between the individuals were observed, and it seemed that the GM did not recover as completely as was observed after the first course. So it seems to be more challenging for GM to recover from the repeated exposures to various antibiotics.

Besides structure, antibiotics can also significantly change the function of the GM, which may further even affect the metabolism of the host. In mice, within 12 hours a single dose of aminoglycoside streptomycin reduced the number of the gut bacteria by 90 %, and within 24 hours this tremendous bacterial reduction was followed by the significant alterations in the levels of fecal metabolites. For example, the level of 17 metabolites involved in the bile acid synthesis and metabolism were decreased in feces (Antunes *et al.* 2011).

2.3 Intestinal immune system and gut microbiota are dependent on each other

The gut serves as an abundant repository for a variety of immune cells (IC). Thus, the so-called intestinal immune system (IIS) is a key player in the host-microbiota crosstalk, in which both the innate and adaptive immunity participate (MacDonald & Monteleone 2005, Maynard *et al.* 2012, Hooper *et al* 2012, Frosali *et al.* 2015, Palm *et al.* 2015). IIS monitors and modulates the GM homeostasis, and microbes reciprocally modify the IIS processes and promote the maturation of the different ICs and the development of the immune functions (Hooper *et al* 2012, Maynard *et al.* 2012, Peterson & Artis 2014, Palm *et al*. 2015). The establishment of the functional IIS in early infancy greatly depends on the presence of the balanced, healthy microbiota (reviewed in Alegre *et al.* 2014).

GM and its structural compartments have a direct impact on the maturation of the intestinal ICs, and each individual will be armed with a unique IIS depending on the type and magnitude of the microbial exposures it encounters during the developmental period (Alegre *et al.* 2014, Peterson & Artis 2014). Thus, aberrant GMC in early life could detrimentally harm the IIS development and affect health later in life (reviewed in Alegre *et al.* 2014). This again emphasizes the importance of the undisturbed development of the GM ecosystem.

Regulatory T cells (T_{reg}) are subpopulations of ICs that are responsible for the differentiation of the self-related patterns from PAMPs, thus enables IS to prevent too excessive reactions, and protect the host from the autoimmune disorders (reviewed in Atarashi & Honda 2011). Especially Th17 cells have emerged as the important mediators

of the host defense and homeostasis at the barrier sites, particularly the intestine, where the greatest number and diversity of the microbes reside (reviewed in Atarashi & Honda 2011). A critical balance exists between the protection of the host from its own microbiota and pathogens and the development of immune-mediated disease. The breaches of the local innate immune defenses provide critical stimuli for the induction of Th17 cell development, and additional cues within these tissues promote the Th17 cell survival and/or plasticity (reviewed in Atarashi & Honda 2011). Normally, this results in eradication of the microbial threat and restitution of the homeostasis. However, when dysregulated, Th17 cells can cause a range of immune mediated diseases either directed against antigens (Ags) derived from the microbiota such as in IBD, or against self-Ags in a range of autoimmune diseases (reviewed in Atarashi & Honda 2011).

The concept of the hygiene hypothesis introduced by Strachan in late 80's states that the development of a balanced immune system (IS) requires a sufficient, early microbial exposure (Strachan 1989). Current urban environment lacks these exposures due to the high use of antibiotics and vaccines together with the improved sanitation, overall hygiene and consumption of processed food and water (Strachan 1989, Haahtela *et al.* 2013). This has led to the increased prevalence of chronic inflammatory and metabolic diseases and autoimmune manifestations in the western world (Haahtela *et al.* 2013). Recently, hygiene hypothesis has been further modified into the biodiversity hypothesis that interconnects the environmental microbial diversity with the host's indigenous microbiota and immunity (von Hertzen *et al.* 2015).

2.3.1 Leaky gut and dysbiosis

Gut epithelial cells provide the host with immediate and transient protection by serving as a physical barrier and triggering an innate immune or proinflammatory response (reviewed in Alegre *et al.* 2014, Belkaid & Hand 2014, Chassaing *et al.* 2014a). The key players in this protection process are intestinal epithelial cells (IECs) and epithelial pattern-recognition receptors (PRRs). Gut epithelium includes four cell types: absorptive enterocytes (IEC), mucus-producing Goblet cells, hormonally active enteroendocrine cells and Paneth cells that lie in the crypts at the base of the small intestine and produce antibacterial peptides (AMPs) or lectins (reviewed in Alegre *et al.* 2014, Chassaing *et al.* 2014a).

The purpose of the mucus, which consists mostly of mucins, is to serve as a GI tract lubricant as well as a protector since it trapps the invading pathogens (Johansson *et al.* 2008). AMPs produced in the Paneth cell such as RegIIIy and alpha-defensin guard the intestinal epithelial layer and participate in the maintenance of the mucosal barrier integrity (Loonen *et al.* 2014). Mice studies have shown that interleukin (IL) -22 production is essential for the RegIIIy production and that IL-22 deficient mice have an aberrant mucosal immunity and are more susceptible to develop MetS and IR (Loonen *et*

al 2014). Interestingly, a recent study showed that a cocktail of 17 gut bacterial species was able to resist the cleansing function provided by the AMPs. Resistant gut bacteria were found from the each phylum commonly present in the gut (Cullen *et al.* 2015)

Healthy intestine maintains a homeostatic state, which shows up as a balance between the effector and T_{reg}s, and the PRRs ability to differentiate the indigenous, conserved microbe-associated molecular patterns (MAMPs) from the pathogenic and harmful ones (Alegre *et al.* 2014). However, the pathogens have evolved in numerous ways in order to disrupt the mucosal barrier integrity and functions. Thus, it is crucial for the host to respond rapidly to the persistently changing conditions (Belkaid & Hand 2014). Microbe recognition and the following straightforward reaction cascades are conserved along the evolution, and eukaryotes possess the PRRs in their intestine. The most studied groups of PRRs are Toll-like receptors (TLRs) and Nod-like receptors (NLRs) that both take care of the intracellular guarding (Akira & Takeda 2004, Chassaing *et al.* 2014a, Frosali *et al.* 2015). In addition, nucleotide-binding and oligomerization domains (NODs), i.e., inflammasomes have recently been shown to be important players in the intestinal homeostasis (Elinav *et al.* 2011, Stienstra *et al.* 2011, Zaki *et al.* 2011). These cytoplasmic multi-protein complexes are assembled from the NLR, caspase-1 and adaptor protein in response to the various damage-and pathogen-associated molecular patterns and promote the maturation and release of the IL-1β and IL-1 (Elinav *et al.* 2011, Zaki *et al.* 2011). The GM-mediated aberrancies in the inflammasome function have been linked to a group of inflammatory bowel diseases (IBD), infection, autoimmune diseases and recently to the obesity and fatty liver disease (Elinav *et al.* 2011, Henao-Mejia *et al.* 2013).

The deficiency in the MAMP genes may cause unwanted changes in the GMC, which may lead to the dysbiotic GM and chronic mucosal inflammation (Cerf-Bensussan & Gaboriau-Routhiau 2010). This further increases the risk of the various disorders. For example, some Crohn's disease patients carry a mutation in the *NOD2*-gene that leads to the non-functional NOD2 (Salem *et al.* 2015). In addition, the continuous overexposure to the heavy loads of PAMPs may also lead to the impaired mucosal barrier integrity, i.e., leaky gut that is a state of the increased permeability of the intestinal epithelium and leads to the increased absorption and translocation of gut-derived toxins, microbial debris and even whole microbes from lumen to the other organs and tissues (Frazier *et al.* 2011). Gut epithelial layer has developed a fine-tuned system for the prevention of the above-mentioned leakage. It includes adherent (E-cadherin) and tight junctions (TJ) and desmosomes (Ulluwishewa *et al*. 2011).

Altered GMC has been recently associated with the increased gut barrier permeability, and increased plasma endotoxin levels have been recognized as a surrogate marker of the bacterial translocation (Cani *et al.* 2007b, Cani *et al.* 2008, Chaissing *et al.* 2014a). One major cause for the decreased mucosal barrier integrity is an unhealthy diet that

seems to have an effect on the translocation of potential endotoxins specifically in the development of obesity and metabolic disorders (Cani *et al.* 2007a, Amar *et al.* 2008). For example in rodent models, the consumption of the HFD has been associated with the unwanted changes in the GM patterns leading to the overall gut dysbiosis (Cani *et al.* 2007b , Cani *et al* 2008).

2.3.2 Toll-like receptors

Toll-like receptors (TLRs) recognize the structurally conserved patterns of bacteria, after which the initiation of an innate immune response begins (reviewed in Könner & Bruning 2011). In the GI tract the innate immune system has been proposed to serve a role in maintaining GM that is beneficial to the host. TLRs are key sensors in the innate immunity and pathogen detection as they are the settled in the interface of the gut epithelium and GM (Könner & Bruning 2011, Frosali *et al.* 2015). To date, altogether 13 different members of TLRs have been identified out of which ten have been found in humans (Frosali *et al.* 2015, Kawai & Akira 2011). TLRs are type I transmembrane proteins that contain Toll-interleukin 1 receptor (TIR) in their intracellular and leucine-rich repeat responsible for ligand recognition in their extracellular domain (Rock *et al.* 1998). TLRs are expressed in various mammalian tissues and cell types such as IECs, hepatocytes, macrophages, Kupffer cells and DCs and each TLR recognizes distinct microbial ligands and as a result a specific inflammatory cascade is triggered (Kawai & Akira 2011).

All signaling pathways (except TLR3) begin with the interaction of adaptor molecule, myeloid differentiating factor 88 (MyD88), with the specific TIR domain of TLR and induction of the cytokine such as TNF-α production. The activation of MyD88 further induces nuclear factor kappa B-driven (NF-κB) pro-inflammatory signaling (Kawai & Akira *et al.* 2011, Frosali *et al.* 2015).

The role of the TLR pathways in many inflammatory diseases including obesity and MetS has been recognized although the exact metabolic consequences of the TLRs' over-expression are not known (Miura *et al.* 2010, Vijay-Kumar *et al.* 2010). Especially the role of TLR2 and TLR4 in the pathophysiology of above-mentioned diseases has been studied (Hardy *et al.* 2012). However, their implication in the obesity-related IR and inflammation is imminent since it has been shown that at least TLR1, TLR5, TLR8, TLR9 and TLR12 are overexpressed in the visceral adipose tissue of the diet-induced obese and genetically obese *ob/ob* mice and the role of TLR2, 4, 5 and 9 has been reported in HFA (Miura *et al.* 2010, Frosali *et al.* 2015).

GM serves as a plentiful source of TLR-activating components and on the other hand TLRs are important in the maintainance of the host's tolerance towards the indigenous microbiota (Rakoff-Nahoum *et al.* 2004). Thus TLRs participate in the regulation of the

assembly of the GMC (Larsson *et al.* 2012). It has been demonstrated that the animals lacking specifically TLR5 possess altered GMC, which further result in diseased host phenotype such as MetS (Vijay-Kumar *et al.* 2010). On the other hand, TLR2/TLR4 double-knock out did not show any effect on GMC in mice (Loh et al. 2008). Thus, the extent to which TLRs influence the GM structure has been disputed since it is possible that the separate breeding and maintenance of knock out and WT animals may act as a source of bias in interpreting the microbiota results (Ubeda *et al.* 2012).

2.3.2.1 Toll-like receptor 2 (TLR2)

TLR2 is expressed by the IECs and intestinal DCs and recognizes a large spectrum of MAMPs such as lipoproteins, lipoteichoic acid and glycoproteins, thus react on both the gram positive and negative bacteria (Cario 2008). TLR2 may form a complex with both the TLR1 and TLR6, which suggests a quite complicated net of responses that further take part in the overall host response (Ozinsky *et al.* 2000). Gut commensals are able to activate TLR2 and this activation leads to the activation of NF-*κB* pathway via the adaptor molecule MyD88. In various inflammatory diseases the increased TLR2 expression and inflammatory cytokine production has been detected thus a low TLR2 expression is important for the intestinal homeostasis. Several mechanisms including the production of anti-inflammatory IL-10 contribute to the maintenance of reasonable TLR2 level in gut (Cario 2008).

TLR2 polymorphisms have been associated with the disturbed immune regulation and thereby with the diseases such as atophy, asthma and IBD (Tunis & Marshall 2014). In addition, TLR2 seems to play a crucial role in the enhancement and function of Th17 cells (Reynolds *et al.* 2010). Notably, the TLR2-induced mechanism of the regulation of T-cell function could enhance microbial clearance and/or increase the risk of autoimmune reactions. However, commensal bacteria use a similar mechanism to enhance the colonization of the gut and thereby establish a host-microbial tolerance.

2.3.2.2 Toll-like receptor 4 (TLR4)

TLR4 that is expressed both on the surface of different ICs such as magrophages, neutrophils and DCs and enterocytes is probably the most studied member of the TLR family, and the appropriate TLR4 signaling is of great importance (Frosali *et al.* 2015). The downstream effects of TLR4 activation vary though the route via the MyD88 adaptor and NF-κB and interferon β release is of major importance (Medzhitov *et al*. 1997, Medzhitov *et al*. 1998, Fukata *et al.* 2005, Frosali *et al.* 2015). However, the triggering of the above-mentioned cascades requires also the activity of co-receptors such LPS-binding protein (LBP) and CD14 (Medzhitov *et al*. 1998, Frosali *et al.* 2015). The TIR domain in TLR4 contains an adaptor that induces interferon (TRIF) –dependent pathways (Medzhitov *et al*. 1998). The

key molecule in the recognition process is MD-2. Commonly TLR4 expression has been considered to be protective for the host. TLR4 expression in IEC is radically increased as a result of acute inflammation that results from disrupted epithelium, which further stimulates the production of inflammatory cytokines such as interferon gamma (INF- γ) or tumor necrosis factor-alpha (TNF-α) leading to he improved condition of injured tissue**.** Increased intestinal TLR4 activity has been found in IBD and acute colitis (Suzuki *et al.* 2003, Singh *et al.* 2005, Fukata *et al.* 2005). However, recent results have suggested also controversial effects where the TLR4 activation exacerbates intestinal inflammation or even inducts mucosal injury (Ungaro *et al.* 2009, Sodhi *et al.* 2010). Interestingly, the role of diet in the TLR4 expression has been highlighted recently. Experimental HFD and low-fiber diet that mimics the current nutritional habits of Western world seems to activate the TLR4 signaling pathway and decrease intestinal integrity via shaping the GMC towards dysbiosis (Cani *et al.* 2008, Frazier *et al.* 2011).

2.3.2.3 Toll-like receptor 5 (TLR5)

TLR5 is a receptor for bacterial flagellin (FLG) that is a principal protein structure of flagella filaments that are responsible for motility and are highly conserved among bacteria (Hayashi *et al.* 2001). Flagella contribute to the virulence of certain bacteria such as *Salmonella* and *Bacillus* and increase their adhesion (Hayashi *et al.* 2001, Ramos *et al.* 2004). FLG-stimulated activation of the TLR5 induces the production of proinflammatory cytokines, such as TNFα, through signaling via the adaptor protein MyD88. Various other proinflammatory and adaptive immune responses are triggered in a response to the TLR5-FLG interaction (Ramos *et al.* 2004, Leifer *et al.* 2014).

TLR5s are expressed abundantly in gut epithelial cells and lamina propria DCs (Uematsu & Akira 2009). In addition, *TLR5* expression has been detected in various cancer cell lines and interestingly localized both on the cell surface and in cytoplasm (Cai *et al.* 2011). The effects of the *TLR5* expression vary a lot depending on the cell type and this receptor influences both on the GM structure and how the IS responses to the bacterial FLG (Honko *et al.* 2005, Chassaing *et al.* 2014b). For example, TLR5s prohibit the bacterial overgrowth in small intestine (SIBO), and on the other hand act as "gatekeepers" since they block the leakage of potential bacterial endotoxins into bloodstream, thus, preventing systemic inflammation (Chabot *et al.* 2008, Chassaing *et al.* 2014a).

TLR5 is known to affect the metabolism of the host, for example impaired function of this receptor in mice has been associated with the traits of MetS (Vijay-Kumar *et al.* 2010). Approximately ten percent of the humans carry a mutation in the TLR5 gene resulting in a complete lack of its function, and as a result express weakened IS that may further predispose to the risk of developing MetS (Chassaing *et al.* 2014b). The role of TLR5 in the intestinal inflammation has recently been studied in *TLR5-null* mice where *TLR5*

was selectively deleted either from the IECs or antigen-presenting DCs (Chassaing *et al.* 2014b). Animals were exposed to the purified FLG, and their response varied depending on the location of the deletion. In addition, IEC *TLR5-null* mice displayed a milder inflammation response than their DC *TLR5-null* counterparts. These mice weighed more, had a higher fat percentage and exhibited elevated blood glucose levels and therefore fulfilled the criteria of MetS. In addition IEC *TLR5-null* mice had higher bacterial load in their feces than the DC model. When both models were exposed to pathogenic *E. coli* the bacterial clearance was significantly delayed in *TLR5 null* enterocytes, and to a lesser extent in the *TLR5-null* DC mice (Chassaing *et al.* 2014b). These results highlight the central role of intestinal TLR5 as a regulator of the immuno-metabolic axis.

2.4 Gut microbiota, obesity and metabolic disorders

Various recent studies conducted in different animal models (rodents, pigs and zebra fish *etc*.) have shown that GM affect obesity and energy metabolism of the host and even the causal relationship has been proven (Bäckhed *et al.* 2004, Velagapudi *et al.* 2010, Tremalori *et al.* 2015). Rodent studies showed already over 15 years ago that GM affects the whole metabolite pool in the host, i.e., the metabolome of the different body fluids such as blood and urine (Nicholls *et al.* 2003). Some metabolites are totally absent from the GF animals, and significantly different metabolome patterns are observed between GF and their conventional (CONV) controls (Wikoff *et al.* 2009, Evans *et al*. 2013). For example, the plasma concentrations of tryptophan and *N*-acetyltryptophan were 40% and 60 % lower and serotonin nearly three-times higher in control compared to GF mice (Wikoff *et al.* 2009).

Selected gut microbial phylogenetic features linked to the obesity and metabolic disorders are collected in Table 2. However, it is quite a challenging task to identify certain indicator bacterial genus or species that seem to unambiguously relate to obesity and metabolic disorders (Walters *et al.* 2014, Stenman *et al.* 2015). For example, the changes in the abundance of certain gut bacteria due to weight-loss may be solely caused by the changes that the intervention causes to the whole GM ecosystem *per se* (Stenman *et al.* 2015). Recent meta-analysis that assembled the GMC results from the five recent studies collected by high-troughput 16S amplicon sequencing concluded that no consistent GM signatures for obesity exist (Walters *et al.* 2014). However, it has been estimated that the different microbial signals may be used with nearly 90 % accuracy in the division of people into obese and lean (Knights *et al.* 2011). Further, it seems that the obese and metabolically impaired phenotype is transmissible via fecal transplantation (FMT) at least in rodents (Bäckhed *et al.* 2004, Turnbaugh *et al.* 2006, Vijay-Kumar *et al* 2010, Ridaura *et al.* 2013). As a result of the colonization with the GM received from the obese, metabolically impaired donor, the altered gene expression in the pathways related to nutrient absorption occurs and thereby altered metabolism in GF animals is observed.

This has direct effects on host phenotype as seen for example as increased adiposity (Bäckhed *et al.* 2004, Velagapudi *et al.* 2010, Vijay-Kumar *et al* 2010). Normally GF animals seem to possess inherently less FM and lower hepatic TG content than their CONV raised counterparts, which may be a result of the decreased lipogenesis and fatty acid (FA) oxidation (Bäckhed *et al.* 2004). In addition, the conventionalization by GM caused a significantly altered lipid metabolism in GF mice, and various correlations have been detected especially when analyzing TGs that are the energy storage form of fat in the body. For example, GF mice showed the altered serum lipidome profile (333 different lipids) after colonization, and the most notable differences were found in the TG fractions (Velagapudi *et al.* 2010). Significant positive correlations between TGs and the uncultured *Ruminococcaceae* related to *R. gnavus* that belong to family in *Clostridium* cluster XIVa have been shown also in humans (Lahti *et al.* 2013)

Table 2. Selected gut microbial groups and individual species linked to obesity and metabolic diseases.

DISEASE/DISORDER	GUT BACTERIA ASSOCIATED WITH THE DISEASED STATE	REF
Obesity & fat mass accumulation	*Firmicutes* phylum	Ley *et al.* 2005 & 2006, Turnbaugh *et al.* 2006, 2009
	Clostridium cluster XIV (mainly *Lachnospiraceae* family members)	Dewulf *et al.* 2011, Verdam *et al.* 2013, Ferrer *et al*. 2013, Tims *et al.* 2013
	E. rectale, C. saccharolyticum	Duca *et al.* 2014
	Lactobacillus acidophilus	Million *et al.* 2012
	Veillonellaceae	Wu *et al.* 2011
	Methanobrevibacter smithii	Schwiertz *et al.* 2010
IR	*Bacteroidetes* phylum	Le Chatelier *et al.* 2013
Metabolic syndrome (dyslipidemia, hyperglycemia & high BP)	*Clostridium* species	Cani *et al*. 2007b, 2008
	Ruminococcus within *Lachnospiraceae*	Lahti *et al.* 2013
	Bacteroides ovatus and *Bacteroides torques*	Zupancic *et al.* 2012
	E. cloacae	Fei & Zhao, 2013
T2D	*Bacteroidetes* phylum, *Proteobacteria* phylum	Larsen *et al.* 2010
	Desulfovibrio, Streptococcus mutans, E. coli, Lactobacillus spp., Egghertella lenta	Karlsson *et al.* 2013
	Akkermansia muciniphila, E.coli	Qin *et al.* 2012
	Lactobacillus spp.	Sato *et al.* 2014
NAFLD	*Bacteroidetes* phylum, g-*Proteobacteria* phylum	reviewed in Wieland *et al.* 2015
	Clostridium coccoides, Lactobacillus	
CVD & Atherosclerosis	*Collinsella*	Karlsson *et al.* 2013, Wang *et al.* 2011

BP=blood pressure, CVD = cardiovascular disease, IR = insulin resistance, NALFD = non-alcoholic fatty liver disease, T2D=type 2 diabetes

2.4.1 Obesity and gut microbiota

Recent body of evidence reports that various phylogenetic signatures exist in GM and genes within the gut microbiome in obesity and metabolic disorders (Table 2). In addition, the metabolic activity of the so-called obese, metabolically impaired microbiome seems to vary significantly from the lean (Turnbaugh *et al.* 2006, Turnbaugh *et al.* 2009). Thus, GM can influence on the host functions that relate to the energy harvest and expenditure, fat storage and eating behavior. Interestingly GF animals are shown to be resistant to HFD-induced obesity (Bäckhed *et al.* 2007) In addition, it has been shown that GF animals live in a state of energy-deprivation, which is characterized by the reduced levels of NADH/NAD^+ balance that leads to lower ATP production (Donohoe *et al.* 2011).

The first findings that suggested a role of GM in obesity were published by Gordon *et al.* already a decade ago (Bäckhed *et al.* 2004, Ley *et al.* 2005, Ley *et al.* 2006). They showed in a series of mice experiments that animals with GM gained more weight and expressed the increased accumulation of TGs into adipocytes, enhanced monosaccharide uptake from the gut and enhanced fatty acid (FA) oxidation in muscle and liver compared to the GF mice after eight-week HFD intervention (Bäckhed *et al.* 2004). In addition, GF mice showed a permanent hepatic and skeletal expression of the adenosine monophosphate (AMP) -activated protein kinase (AMPK) that stimulates the catabolic, i.e., ATP-producing pathways under stressful conditions thus monitoring the energy status of the cell. The absence of AMPK in GF mice may at least partly explain their resistance to HFD induced obesity and IR (Bäckhed *et al.* 2004, Bäckhed *et al.* 2007). Further, the presence of the GM suppressed intestinal expression of fasting-induced adipose factor (Fiaf), which is a glycosylated protein produced by the enterocytes, adipocytes and hepatocytes (Kersten *et al.* 2005). GM regulates the expression of Fiaf inhibits. Its inhibition triggers lipoprotein lipase (LPL) in the adipose tissue, which further leads to reduced triglyceride and FA accumulation (Bäckhed *et al.* 2004, Mandard *et al.* 2006).

Gordon *et al.* demonstrated also for the first time that the transmission of GM from CONV mice into the intestine of GF mice promoted the absorption of monosaccharides from the intestines, affected the activity of certain enzymes present in the IECs and increased hepatic lipogenesis resulting in increased anabolic metabolism without increase in food consumption (Bäckhed *et al.* 2004). These pioneering findings led to the efficient energy extraction (EEE) hypothesis suggesting that the GM could contribute to obesity by enhancing the recovery of energy from the diet especially through the colonic breakdown of dietary fiber and other complex polysaccharides that remain undigested in the small intestine. This extra energy causes changes in the metabolism of the host. It is estimated that nearly ten percent of our daily energy gain is derived from

the bacterial digestion of dietary fiber – an energy source that our own digestion cannot utilize (Cummings & MacFarlane 1997).

NGS approaches have supported the EEE hypothesis, since the obese gut microbiome seems to display an increased expression of genes responsible for the breakdown of complex polysaccharides and pathways related to starch, sucrose and butanoate metabolism leading to more effective release of calories from the diet to the host (Turnbaugh *et al.* 2006, Turnbaugh *et al.* 2008). Recent metagenomic studies have also reported that the obese and metabolically impaired phenotype harbors lower bacterial diversity described as gene richness compared to the lean phenotype (Turnbaugh *et al.* 2009, Larsen *et al.* 2010, Le Chatelier *et al.* 2013). However, recent meta-analysis of five original publications could not verify that the overall GM diversity could distinguish the obese and lean subjects (Walters *et al.* 2014). Further, only one fourth of the obese subjects actually harbored a decreased microbial richness in the gut, thus it seems that low gene richness in microbiome correlates only with metabolic disorders (Le Chatelier *et al.* 2013).

2.4.1.1 Examples of bacterial groups and species that has been associated with obesity and increased body fat accumulation

The role of *Clostridium* cluster XIVa within *Firmicutes* phylum in obesity has recently been suggested (Duca *et al.* 2014). Already the first metagenome analysis of leptin-deficient *ob/ob* mice cecal content showed the abundance of genes encoding enzymes that are involved in breaking down indigestible dietary polysaccharides such as glycoside hydrolases in the obese microbiome (Turnbaugh *et al.* 2006). Further KEGG pathway analysis revealed the enrichment of pathways related to starch, sucrose, galactose and butanoate metabolism in the obese microbiome, paralled with increased concentrations of butyrate and acetate in the cecum. One member of the *Clostridial* cluster XIVa, *E. rectale*, that is a known butyrate-producer, contains over 40 different glycoside hydrolases in its genome from which most are specialized to the degradation of dietary starches (Turnbaugh *et al.* 2006). When the *ob/ob* microbiome was compared against the whole genome of *E. rectale,* the abundance of *E. rectale*-type glycoside hydrolases was observed, suggesting that a high amount of this bacterium in the gut may lead to too efficient energy extraction leading to increased fat accumulation in the body (Turnbaugh *et al.* 2006, Turnbaugh *et al.* 2009). *E. rectale* belongs to the *Lachnospiraceae* family within *Clostridial* cluster XIVa. Various members of this bacterial group have been recently associated with the obese and non-healthy phenotype in humans (Table 2, Santacruz *et al.* 2009, Verdam *et al.* 2013, Ferrer *et al.* 2013, Tims *et al.* 2013, Walters *et al.* 2014).

Many studies report increased amounts of potentially pathogenic bacteria in the GM of overweight/obese subjects or rodents on HFD. For example, greater fecal amount of the gram-positive opportunistic pathogen *Staphylococcus aureus* has been reported both in obese, pregnant women and overweight children (Kalliomäki *et al.* 2008, Santacruz *et al.* 2010). Simultaneously increased proportions of gram-negative *Enterobacteriaceae* were detected (Santacruz *et al.* 2010). Recent case-report describes the seriously disrupted fecal microbiota that contained the gram-negative *Enterobacter* genus up to 35 % proportion in morbidly obese Chinese suffering from both hyperglycemia and hypertension. Volunteer was able to lose 50 kg of weight as a result of a 23-week diet intervention, and simultaneously the proportion of *Enterobacter* dropped to the non-detectable level (Fei & Zhao, 2013)

The existence and prevalence of methanogenic *Archaea* in the obese microbiome has been suggested (Zhang *et al.* 2009). For example, comparison of three study groups revealed that the obese subjects had significantly more methanogens in their feces than the normal weight or post-gastric bypass subjects. In the obese group, coincidental existence of the members of *Prevotellaceae* that are known hydrogen producers was observed (Zhang *et al.* 2009). This suggests that the interspecies hydrogen transfer may be one of the mechanisms behind the increased energy uptake in obese individuals since the methanogens are able to oxidize hydrogen, which enhances the SCFA production of fermentative bacterial species leading to more effective energy extraction (Samuel & Gordon 2006, Samuel *et al.* 2007). *Methanobrevibacter smithii* has also been reported to be associated with the lean phenotype in humans (Goodrich *et al.* 2014b, Million *et al.*, 2012). However, *M. smithii* was much more abundant in the patients with anorexia nervosa compared to the healthy lean people suggesting that it may rather act as an indicator of energy status (Armougom *et al.* 2009).

Mollicutes class of *Firmicutes*, especially *Eubacterium dolichum,* has been shown to have an influence on the host energy balance (Turnbaugh *et al.* 2008). The inoculation of this bacterium to the GF mice on carbohydrate-rich Western diet resulted in a greater adiposity than in their lean counterparts on normal chow. Metagenomic analysis revealed that *E. dolichum*-rich GM harbor a versatile set of genes encoding for phosphotransfarase system for fructose and mannose. In addition, the ability to efficiently ferment various single sugars provides a competitive advantage in high-fat, high-sugar milieu (Turnbaugh *et al.* 2008).

2.4.1.2 Examples of bacterial groups and species that has been associated with good metabolic health

Few recent cross-sectional studies have addressed the beneficial role of *F. prausnitzii* in adiposity and metabolic health (reviewed in Stenman *et al.* 2015, Miguel *et al.* 2015).

Greater *F. prausnitzii* abundance has been associated with the metabolically healthy phenotype in humans (Hvistendahl 2012, Le Chatelier *et al.* 2013, Wilson *et al.* 2014). Recent case-report showed that the significant weight loss (20 kg per 24 months) and simultaneous improvement of metabolic health associated with the increase of *F. prausnitzii*. During the intervention period, diet rich in whole grains and Chinese bitter melon was consumed, and thus dietary factors may at least partly explain the bloom of *F. prausnitzii* during the weight-loss (Hvistendahl 2012). In addition, a depleted amount of *F. prausnitzii* has been associated with the traits of MetS and SLGI in humans (Zupancic *et al.* 2012, Verdam *et al.* 2013, Clarke *et al.* 2014, Remely *et al.* 2015a). It seems that *F. prausnitzii* possess anti-inflammatory properties and improves gut barrier, and its function in the gut may improve the state of GM dysbiosis and further metabolic endotoxemia (Carlsson *et al.* 2013). Finally, a significant negative association between BMI and *F. prausnitzii* was also verified in a recent meta-analysis suggesting that this particular species could serve as indicator taxa for good metabolic health in the future (Walters *et al.* 2014).

Recently a causal role of mucin-degrader *A. muciniphila* in the weight-loss and maintenance of good metabolic health has been suggested based on studies conducted in mice (Everard *et al.* 2013, Shin *et al.* 2014). In addition, the greater abundance of *Akkermansia* has been associated with the increased gut microbiome gene richness, which is a feature recently linked to th lean phenotype in humans (Le Chatelier *et al.* 2013, Dao *et al.* 2015). Recent human studies have also shown that the abundance of *Akkermansia* was decreased in diabetics, and increased during the intervention with successful weight-loss (Zhang *et al.* 2013, Remely *et al.* 2015a). However, opposite finding has been reported by a Chinese group that showed that *Akkermansia* was actually enriched in the T2D patients compared to the healthy controls (Qin *et al.* 2012). It seems that both *Akkermansia* and *F. prausnitzii* are able to improve the gut barrier integrity both *in vivo* and *in vitro* (Everard *et al.* 2013, Reunanen *et al.* 2015).

One new interesting candidate for the indentification of the metabolically healthy GM is the *Christensenellaceae* family that was recently associated with lean phenotype in one experiment conducted in humans (Goodrich *et al.* 2014b). In addition, *Christensenellaceae* supported the successful weight loss in mice. GF mice that received fecal transplant low in *C. minuta* gained significantly more weight, at a significantly faster rate, than those conventionalized with the feces rich in *C. minuta* (Goodrich *et al.* 2014b).

2.4.1.3 The effect of antibiotics on overall GMC and adiposity

The increased usage of antibiotics has recently been linked to the obesity epidemic (reviewed in Cox & Blaser 2015, Nobel *et al.* 2015). It is well known that even short-term antibiotic treatments affect GMC and function quite profoundly, and as a result negative metabolic effects may affect the host (Dethlefsen & Reiman 2011). For example in the adult study population a long-term course of vancomycin has been linked to an increased level of obesity (Thuny *et al.* 2010). In addition, too frequent use of antibiotics in early life seems to have long-term effects on GM diversity, which may further lead to a higher incidence risk of childhood obesity (Kalliomäki *et al.* 2008, Ajslev *et al.* 2011, Nobel *et al.* 2015). Few recent findings have linked the usage of antibiotics to the causal relationship between metabolism and GM. For example an oral vancomycin significantly damaged the insulin sensitivity in males fulfilling the criteria of MetS. Impairment occurred via altered bile acid dehydroxylation and simultaneously changed the GMC (Vrieze *et al.* 2014). In young mice, the usage of subtherapeutical doses of antibiotics altered the GMC, copies of key genes involved in the metabolism of carbohydrates and SCFA production profiles (Cho *et al.* 2012). Interestingly, the chosen drug cocktails mimicked the actual antibiotic courses that are regularly used as growth promoters in the animal production in the US. Treated mice had over 15 % higher body FM than their controls, and expressed hepatic lipolysis and increased hormone levels (Cho *et al.* 2012). Interstingly in the treated mice the bloom of *Lachnospiraceae* were observed, which further led to increased *Firmicutes/Bacteroidetes* (F/B) ratio that was associated with the increased adiposity (Cho *et al.* 2012).

2.4.1.4 The role of gut microbiota metabolites

The various metabolites produced by gut bacteria are crucial for the development and maintenance of the mucosal IS and epithelial barrier and the overall gut homeostasis. In addition, these molecules seem to contribute to the obesity and energy metabolism (Samuel *et al.* 2008, Dewulf *et al.* 2011, Hartstra *et al.* 2015). For example SCFA bind and activate G protein–coupled receptors (GPR) 41 and 43. GPRs regulate the metabolic rate of the host, and are expressed in IECs and also in the liver and adipose tissue (Samuel *et al.* 2008; Tazoe *et al.* 2008, Allin *et al*. 2015). As a result of the butyrate activation, secretion of gut peptide YY (PYY) and glucagon-like peptide 1 (GLP1) into circulation occurs (Gao *et al.* 2009, Tolhurst *et al.* 2012, Allin *et al*. 2015, Figure 5). Acetate and propionate are further utilized as precursors in hepatic gluconeogenesis and lipogenesis (Allin *et al*. 2015, Figure 5). If GPR41 function is impaired, PYY production is reduced, which further leads to a decreased extraction of energy from the diet. As a result a prolonged gut transit time is observed (Samuel *et al.* 2008).

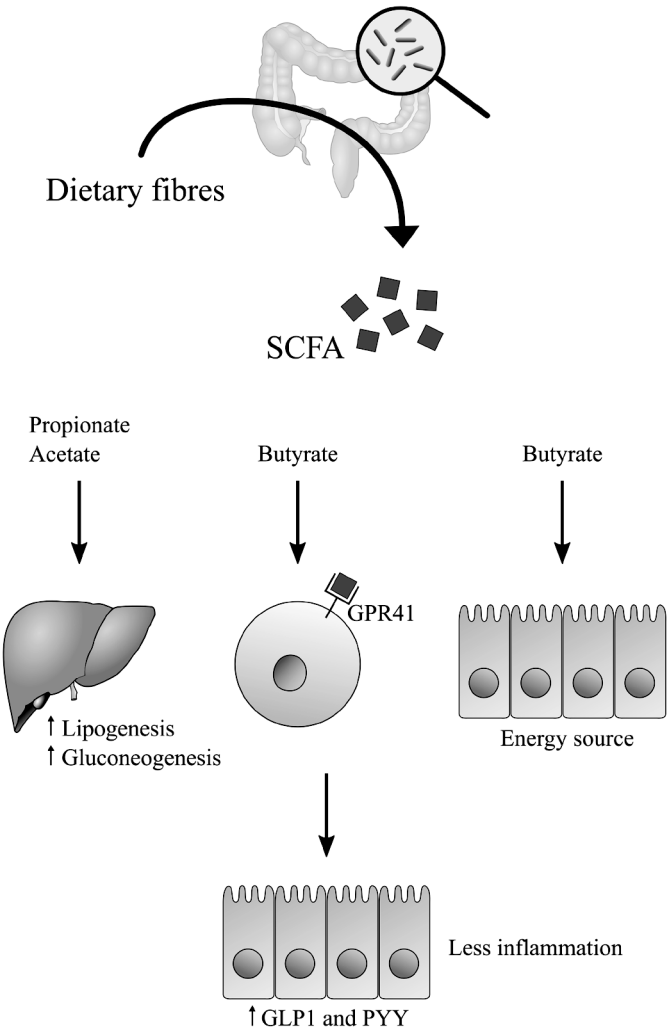

Figure 5. SCFA and host metabolism. Modified from (Allin *et al*. 2015). GLP1 = glucagon-like peptide 1, GPR = G protein–coupled receptor, PYY = gut peptide YY

Butyrate is one of the most studied health-promoting molecules produced by the GM, and a lack of this metabolite in the gut has been associated with various inflammatory intestinal diseases (Hamer *et al.* 2008, Sokol *et al.* 2008, Sokol *et al.* 2009). It seems to have a positive influence on the host energy metabolism (Brahe *et al.* 2013). Butyrate has various functions - it serves as an energy source for the colonic epithelial cells, improves the integrity of the gut barrier and alleviates the inflammation (Hamer *et al.* 2008, Sokol *et al.* 2008). For example, experimental oral sodium butyrate treatment has been shown to cause systemic anti-inflammatory effect both in CD and UC patients (Vernia *et al.* 2000, Di Sabatino *et al.* 2005).

Both butyrate and propionate may affect the gluconeogenesis that occurs in the gut. Butyrate contributes beneficially to the overall glucose metabolism in the body by activating the event called intestinal gluconeogenesis (IGN) through the cAMP-dependent mechanism (De Vadder *et al.* 2014). Propionate utilizes different mechanism and thus affects IGN via direct gut-brain neural circuit including GPR41 (De Vadder *et al.* 2014). Butyrate seemed to improve insulin sensitivity and increase energy expenditure via enhanced mitochondrial activity in mice (Gao *et al.* 2009). Further, it was shown that butyrate inhibits histone deacetylases, which may lead to the increased expression of transcriptional activator PGC-1a that has been associated with the increased FA oxidation (Gao *et al.* 2009). However, to date these effects have not been conferred in humans. In addition, butyrate contributes to the maintenance of gut epithelial integrity, and thus prevents SLGI, which occurs when endotoxic and/or antigenic compounds are translocated from the gut into the bloodstream (Brahe *et al.* 2013).

Further, animal studies have shown that butyrate participates in the intestinal production of neurotransmitter serotonin, which further enhances satiety but also regulates the gut integrity (Gill *et al.* 2013). Interestingly, low levels of hypothalamic serotonin receptors have been associated with the obese phenotype in rats (Ratner *et al.* 2012). Then again, serotonin levels in the gut seem to be affected by GM (Wikoff *et al.* 2009). This phenomenon has been proven in studies that evaluated the effects of Roux-en-Y gastric bypass (RYGB) on metabolism and GMC both in mice and humans (Zhang *et al.* 2009; Furet *et al.* 2010; Liou *et al.* 2013; Tremalori *et al.* 2015). When fecal transplant from the RYGB-treated mice were transferred to the diabetic mice, significant weight loss and improved glucose and lipid metabolism were observed, and at the same time the number of butyrate-producing bacteria in gut the increased (Liou *et al.* 2013). Alterations observed were independent of the caloric restricted diet and concurrent weight loss.

2.4.2 Gut microbiota, metabolic syndrome and type 2 diabetes

MetS is often associated with ongoing IR and SLGI, in which a variety of inflammatory molecules such as cytokines are constantly produced by various ICs (Hotamisligil 2003; Esser *et al.* 2014; Guida & Venema 2015). For example as macrophages infiltrate into the obese adipose tissue, the production of pro-inflammatory factors begins (Weisberg *et al.* 2003, Xu *et al.* 2003). This further predisposes various peripheral tissues to the IR, which is manifested as liver failures to inhibit the glucose production, release of the free FAs from the adipose tissue and simultaneously reduced uptake of glucose by the adipose tissue and skeletal muscle occurs (Reaven 2005, Amar *et al.* 2008). Currently it seems that GM contributes to the obesity-associated inflammation and therefore has an important role in the development of traits of MetS (reviewed in Hartstra *et a*l. 2015). For example an elevated F/B ratio has been reported in IR, and thus GM seems to play

an important role in this prediabetic condition (Ley *et al.* 2005, Tremalori & Bäckhed 2012).

2.4.2.1 Gut microbiota and adipose tissue inflammation

It seems that LPS, a cell wall outer membrane's component from the gram-negative members of GM, is involved in the trigger and maintenance of SLGI (Cani *et al.* 2007a,b). This state that is linked also to the increased gut permeability was named metabolic endotoxemia by Cani and colleagues almost ten years ago (Cani *et al.* 2007a,b). In blood LPS molecules translocated from the gut bind the specific LPS-binding proteins, and further activate TLR4 on the surface of macrophages and other CD14 expressing cells. As a result, the proinflammatory NF-*k*B pathway is induced, and the production of IL-1 and TNF is further stimulated. Simplified overview of the metabolic endotoxemia is representeded in Figure 6**.**

HFD seem to act as an enhancer of LPS translocation from the gut into circulation (Cani *et al.* 2007a, Erridge *et al.* 2007). The increased hepatic expressions of IL-1, IL-6 and TNF-α as well as an inflamed adipose tissue have been observed even if LPS was given as continuous subcutaneous infusion via implanted osmotic minipump. Thus both HFD and LPS contribute to the development of metabolic endotoxemia (Cani *et al.* 2007a,b). CD14 knockout mice do not express the increased inflammation marker gene levels in their adipose tissue or unfavorable changes in the phenotype. HFD seems to specifically reduce the abundance of *Bifidobacterium* in the gut, which may further explain the simultaneously perceived impaired mucosal barrier function (Cani *et al.* 2007b, Cani *et al.* 2008). However, the abundance of LPS in the gut not necessarily correlates with the rate of endotoxemia. A recent metagenomic study reported that the several genes from the LPS biosynthetic pathway actually decreased in the response to the HFD. This questions at least to some extent the existence of a direct relationship between the LPS synthesis potential provided by GM and metabolic endotoxemia (Everard *et al.* 2014). Besides TLR4, it has also been proposed that TLR5 has a role of in signaling pathways affecting the development of MetS as observed in the studies utilizing *Tlr5*-deficient mice (Vijay-Kumar *et al.* 2010).

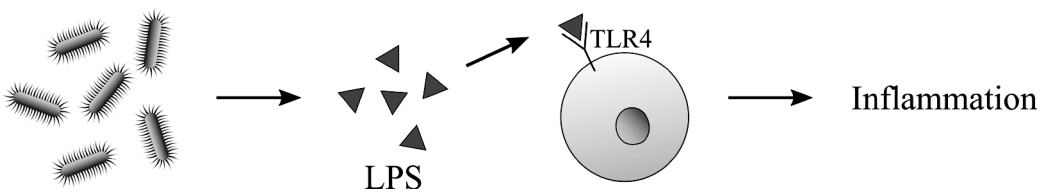

Figure 6. Simplified schematic presentation of metabolic endotoxemia. LPS of gram-negative bacteria-origin binds to the Toll-like TLR4, which activates pro-inflammatory signaling pathways resulting in SLGI and thus decreased insulin sensitivity in host. Modified from Allin et al. 2015.

2.4.2.2 Endocannabinoid system (eCB) in regulation of gut-adipose tissue axis

It has been proposed that the so-called endocannabinoid system (eCB) acts as a mediator between GM and the metabolism of the adipose tissue (Muccioli *et al.* 2010). Stimulated eCB system tone is increased in obesity and IR, enhances plasma LPS levels and worsens the gut barrier (Guida & Venema 2015). The eCB consists of two receptors, namely CB_1 and CB_2 (Muccioli *et al.* 2010). Both eCB receptors are expressed in the tissues and organs that are important for the maintenance and regulation of the energy balance: CB_1 is present in the brain, adipose tissue and skeletal muscle while CB_2 in IC (Furuya *et al.* 2012, Kushner 2012). The best-characterized eCB molecules are 2-arachidonoylglycerol (2-AG) and anandamide (AEA) both of which are released to reach their receptors immediately after their production (Cani *et al.* 2014). Both previously mentioned eCBs are degraded by FA amide hydrolase (FAAH) and monoacylglycerol lipase (Muccioli *et al.* 2010). The eCB system seems to modify the glucose metabolism via affecting GLUT-4 expression in the adipose tissue (Furuya *et al.* 2012). In adipocytes, the inhibition of CB_1 receptor activity by a specific agonist, increased the expression of GLUT-4 that is a well-studied glucose transporter, the low expression levels of which have been associated with IR.

Recently the role of eCB in GM-related metabolic endotoxemia and adipogenesis were evaluated in mouse models and *in vitro* in Caco-2 cell culture that models epithelial cell function (Mucciolo *et al.* 2010). When the function of CB_1 was blocked in *ob/ob* mice by a selective antagonist, reduced body FM and plasma LPS levels as well as an improved gut barrier function were observed. The latter effect was further ascribed to an altered localization and abundance of two TJ proteins ZO-1 and occludin. The Caco-2 cell stimulation with both LPS and CB_1 agonist resulted in decreased levels of TJ proteins. Epithelial barrier integrity was measured by transepithelial electrical resistance (TEER), and when CB_1 receptor antagonist were included in the experiments, both TJ protein expression levels and epithelial barrier integrity were normalized to the level of controls. Thus eCB seems to regulate gut permeability, and the key component is CB_1. Finally, when obese mice were fed with prebiotics, the decreased body fat as well as AEA levels and CB_1 expression in adipose tissue were observed. It seems that the dietary modification of GM is able to set the tone of eCB system (Mucciolo *et al.* 2010, Guida & Venema 2015).

2.4.2.3 Gut microbiota in type 2 diabetes

As in obesity, predisposing factors for T2D include genetic vulnerability and unhealthy lifestyle. In addition, recent accumulating evidence suggests that GM may mediate or modulate the impact of lifestyle factors thus enhance the risk in the development of T2D (reviewed in Allin *et al.* 2015, Delzenne *et al.* 2015). It seems that T2D patients

exhibit at least moderately altered GMC compared to their healthy counterparts (Larsen *et al.* 2010, Qin *et al.* 2012, Karlsson *et al.* 2013, Sato *et al.* 2014, Sepp *et al.* 2014). For example, T2D is associated with the reduced levels of *Bifidobacteria* and increased levels of gram negative bacteria capable of endotoxin production (Larsen *et al.* 2010, Wu *et al.* 2010, Qin *et al.* 2012, Karlsson *et al.* 2013). Recent large-scale metagenomic studies conducted in China (*n*=345) and Sweden (*n*=145, only elderly women) both reported the decreased abundance of the butyrate-producers such as *F. prausnitzii* in T2D patients (Qin *et al.* 2012, Karlsson *et al.* 2013). Same kind of results has been reported in a Japanese study in which the reduced levels of *C. coccoides* group in T2D patients (*n*=50) compared to healthy controls were observed (Sato *et al.* 2014).

Neither the Chinese nor the Swedish study population expressed significant difference in the F/B ratio or bacterial richness and diversity between the patients and the controls. However, there are many discrepancies between the two studies that may be explained by age, ethnicity and differences in diet. In the Chinese cohort including both men and women the abundance of opportunistic pathogens such as *E.coli* was increased in the T2D group. This result is in line with a recent Danish study reporting higher levels of *Betaproteobacteria* that associated positively with blood glucose level in diabetic group (Larsen *et al.* 2010). Swedish study reported increased levels of *Lactobacillus* spp. in T2D group and this bacterium correlated positively with the fasting glucose and glycosylated haemoglobin that is a long-term biomarker of blood glucose level (Karlsson *et al.* 2013). Further in elderly population *Bacteroides* abundance negatively correlated with the high blood glucose level (Sepp *et al.* 2014).

Metagenomic studies have also revealed that some functions of microbiome are altered in the T2D patients compared to controls (Qin *et al.* 2012, Karlsson *et al.* 2013). For example reduced capacity for functions related to the cell motility, butyrate synthesis and vitamin metabolism can be seen in T2D. On the contrary, T2D microbiome possesses increased gene content related to the carbohydrate metabolism and membrane transport of sugars and branched amino acids (Qin *et al.* 2012, Karlsson *et al.* 2013).

Various factors that affect simultaneously both GMC and risk of T2D must be taken into account in the statistical analysis and interpretation of the results. One confounding factor besides ethnicity and diet that may affect the GM results is the intake of medications although to date the information concerning the specific effects of medication remain scarce (Allin *et al.* 2015). Medication clearly has an effect since for example a metformin treatment was shown to increase the abundance of *A. muciniphila* in HFD-fed, diabetic mice (Shin *et al.* 2014). A Swedish study reported that the T2D patients taking metformin had increased abundance of *Enterobacteriaceae* indicating that the information about the drug use should be taken into account in the GM assessments in the future (Karlsson *et al.* 2013, Allin *et al.* 2015).

2.4.3 Excessive hepatic fat accumulation and gut microbiota

Non-alcoholic fatty liver disease (NAFLD) is a chronic condition characterized by the hepatic fat accumulation usually combined with IR and low-grade inflammation without excessive alcohol consumption (Yki-Järvinen 2014). NALFD is sometimes considered as one of the phenotypes of MetS, and its pathogenesis is multifactorial event (Bieghs & Trautwein 2013). Obese individuals accompanied with dyslipidemia and/or IR are at the greatest risk of developing NAFLD that has become the most frequent cause of chronic liver disease in Western countries. The occurrence is estimated to be nearly 15-30% in general population and up to 80% among obese individuals and the incidence is rising. It affects both children and adults with particular risk factors including the genetic susceptibility, consumption of the obesogenic diet and physical inactivity (Vernon *et al.* 2011, Milic *et al.* 2012). NAFLD has been suggested to associate with both T2D and cardiovascular disease (CVD).

Fatty liver possess abnormal metabolism illustrated by the overproduction of both lipids and glucose that together with the increased levels of imported lipids exceeds the catabolism in hepatocytes. In addition, simultaneous dysfunction of the insulin signaling may occur. In the long run, liver fails to export the excess molecules and maintains impaired mitochondrial β-oxidation of FAs (Miura *et al.* 2010). While to date the metabolic consequences of NAFLD are well documented, the complex pathophysiology behind the diseased state has not yet been fully elucidated (Yki-Järvinen 2014). Constant elevated flux of free FAs from the increased visceral adipose tissue into liver may lead to NAFLD. In addition, some genetic predisposition exist. Recently scientific studies have concentrated on the inflammatory processes and suggested that for example mitochondrial dysfunction, oxidative stress and/or lipid peroxidation predispose liver to the state of inflammation (Besse-Patin & Estall 2014).

NALFD progresses gradually usually without substantial outward symptoms (Yki-Järvinen 2014). The early stages include abnormal hepatic fat accumulation (HFA), and might be a quite benign state. However, at the molecular level the constantly increased assortment of hormones derived from the dysfunctional adipose tissue with the consequent oxidative stress and fibrogenesis may lead the steatohepatitis (NASH) and at worst to liver cirrhosis, which is a state of chronic inflammation and accumulation of collagen and scarring (Chalasani *et al.* 2012, Yki-Järvinen 2014, Bieghs & Trautwein 2013). Cirrhosis may further predispose to the hepatocellular carcinoma (Yki-Järvinen 2014). The state of mild steatosis is determined by the cut-off value of > 5% fatty hepatocytes in the liver, and fortunately only one fifth of the individuals that fulfill criteria will end up developing the progressive form of the disease (Yki-Järvinen 2014).

Recently GM has emerged as a potential novel factor causing the onset and progression of NAFLD (Miura *et al.* 2010, Henao-Mejia *et al.* 2013, Imajo *et al.* 2014). Rodent studies have demonstrated the convincing evidence for a central role of aberrant GM in abnormal HFA, and the suggested mechanisms include for example impaired gut barrier and an aberrant host immune response (reviewed in Aqel & DiBaise, 2015, Quigley & Monsour 2015). In addition, the beneficial effects of the microbiota modulation by probiotics or prebiotics on preventing the progression of disease have been shown (Federico *et al.* 2015). However, the evidence from human studies is scarce as association of GM with HFA has been documented mainly in cross-sectional studies, and the exact mechanisms are still unclear (Quigley & Monsour 2015).

2.4.3.1 Proposed mechanisms of gut-liver axis in the pathogenesis of NALFD

The existence of the functional gut-liver axis has been recognized for almost 100 years now (Abu-Shanab & Quigley 2010). Gut and liver are interconnected via an anatomical joint portal vein through which the blood containing digested nutrients from small intestine floods into the liver (Abu-Shanab & Quigley 2010, Federico *et al.* 2015). Besides compounds originated from the diet, blood derived from the small intestine includes bile, hormones and inflammatory and other signal molecules maintaining continuous bidirectional cross talk between the two organs (Federico *et al.* 2015). Thus, various by and end products originating from the GM fermentation, actual bacterial components such as LPS, peptidoglycan and bacterial DNA, and even intact bacteria flow into the liver and affect directly its function (Chassaing *et al.* 2014c). For example, many byproducts of the GM fermentation process such as ethanol, acetaldehydes and ammonia are known to be toxic to liver (Abu-Shanab & Quigley 2010). In addition, various indirect effects of GM on liver function exist.

NAFLD-prone mice seem to have aberrant GM structure characterized by the small intestinal bacterial overgrowth (SIBO) and simultaneous reduction in the expression levels of intestinal TJ proteins (Brun *et al.* 2007). In addition to the obese individuals, SIBO has been reported to be a common feature of liver cirrhosis patients (Kapil *et al.* 2015, Abu-Shanab & Quigley 2010). The release of the bile acid into the small intestine after eating seems to prohibit the bacterial overgrowth by the activation of farnesoid X receptor (FXR) and further protecting jejunum and ileum from too high bacterial load (Inakagi *et al.* 2006, Schiller 2007). Experimental blockage of the flux of bile acids from the liver into the small intestine induces SIBO accompanied by the increased intestinal permeability and systemic infection (Inakagi *et al.* 2006). Hence, the liver seems to directly affect GMC in the distal small intestine.

One of the most studied examples of indirect effects of GM on the liver is the role of increased TLR-related inflammatory stimulus (Rivera *et al.* 2007, Spruss *et al.* 2009,

Pinzone *et al.* 2012, Miura & Ohnishi 2014). Healthy liver is able to maintain a high tolerance againstthe TLR-activating bacterial ligands whereas fatty liver upholds ongoing TLR activation (Miura & Ohnishi 2014). Continuous induction of the hepatic TLRs further induces downstream pathways, and finally production of various cytokines and chemokines occurs, which leads to the development of chronic inflammation. Further, TLR knockout mice seem to be resistant to liver inflammation and fibrosis. To date, the role of at least TLR2, TLR4 and TLR9 in NALFD has been acknowledged (Szabo *et al.* 2005, Miura *et al.* 2010). The most studied example is probably the leakage of LPS from gut which triggers hepatic TLR4 cascade and leads the increased IR, steatosis and further NASH (Kapil *et al.* 2015).

It has been shown in mice that dietary choline deficiency predisposes animals to HFA (Kirsch *et al.* 2006). Choline is a water-soluble molecule classified into complex B vitamins that serves a variety of important biological functions in the body. It is for example a precursor for neurotransmitter acetylcholine and phospholipids that build up cell membranes. Choline metabolism may be disrupted due to aberrant GMC caused by HFD, which further enhances the development of the NALFD in genetically predisposed mice (Dumas *et al.* 2006). Further it has been shown that GF mice are unable to perform the conversion of choline into di- and trimethylamine (TMA), thus certain bacterial members of GM are prerequisite for functional choline metabolism.

2.4.4 Cardiovascular diseases (CVD) and atherosclerosis

High blood pressure (BP), i.e., hypertension (HT), is one of the five traits of MetS and a risk factor for both cardiovascular disease (CVD) and T2D (Alberti *et al.* 2005). As both HT and GMC are influenced by the genetic and environmental factors it is no surprise that the association between these two has been recently addressed (Howitt & Garrett 2012, Tang *et al.* 2013, Yang *et al.* 2015). Recently Yang *et al.* (2015) reported the decreased microbial richness and diversity, lower amounts of *Bifidobacteria* and increased F/B ratio in the spontaneously hypertensive rats (mean 148 mmHg) compared to their controls. Deeper taxonomical analysis revealed that the HT rats had significantly more lactate-producing bacteria (*Streptococcus* & *Turicibacter*) and less butyrate-and acetate-producers than the controls. In addition, the fecal microbiota structure of the human subjects with high systolic BP (mean 144 mmHg, n=7) and HT treatment exhibited the same kind of features as rat models: reduced Chao richness in bacterial species level and Shannon diversity compared to the healthy controls (n= 10). The two subject populations also generated distinct, separate clusters in PCoA plot, which indicates the significantly different GM structures. So high BP seems to associate with the unbalanced GM and further impacts distorted metabolite production.

GM metabolizes certain dietary nutrients such as phosphatidylcholine (PC) and L-carnitine into proatherosclerotic trimethyl metabolites (Wang *et al.* 2011). This may further lead to the development of atherosclerosis and CVD in obese rodents and humans. PC and L-carnitine, via the formation of TMA-N-oxide that possess trimethylamine (TMA)-moiety, take part in the development of CVD (Koeth *et al.* 2013, Tang & Hazen 2014). Thus, GM possesses many endocrinological functions, and may serve as an active participant in the development of atherosclerosis and CVD.

2.4.5 Gut microbiota modification as a treatment strategy in metabolic diseases

In the future, targeted GM modulation may be a promising new treatment concept in obesity, MetS and NALFD (Lankelma *et al.* 2015). The therapeutic aspect of GM arises from the fact that traditionally we have not been able to change the genes of the host, but however can change our microbial community. Despite the natural resilience of GM, it is possible to modify its structure and function even at adult age. Naturally the simplest way for an individual to affect and change the GMC is the changing of the dietary habits in order to improve the metabolic health status. In addition, many recent studies concerning the improvement of metabolic health have suggested that the bacterial therapies such as probiotics, prebiotics or even fecal transplant (FMT) will be utilized in personalized manner in the future. The examples of various different bacterial therapies in humans based on recent reviews and meta-analysis has been collected in Table 3.

Probiotics, especially various *Lactobacillus* and *Bifidobacterium* strains, have been suggested to have favorable effect on the weight loss and energy metabolism (Ishimwe *et al.* 2014). However, most studies have been performed in rodents or *in vitro* conditions thus there is a lack of proper human intervention studies (Gerard 2015). It seems that the probiotic effects on weight and metabolism are strictly species and strain dependent (Million *et al.* 2012). For example, *L. gasseri* seem to associate in the reduction of body weight both in rodents and in obese humans (Million *et al.* 2012). On the contrary, administration of another member of *Lactobacillus* spp., namely *L. acidophilus* actually resulted in significant weight gain both in rodents and in humans (Million *et al.* 2012). Recent systematic review summarized the evidence received from the clinical trials in humans and focused on the usage of probiotics as a treatment for the weight loss and reported that only four original studies that were randomized controlled trials (RCTs) met their inclusion criteria (Park & Bae 2015). The meta-analysis showed no significant effects of probiotics on body weight, BMI or weight loss. Since the number of trials included in the analysis was so low, no definitive conclusions were drawn (Park & Bae 2015). To date no official recommendations about the therapeutical usage of probiotics in obesity exists (Floch 2014).

The most studied prebiotics, i.e., dietary non-digestible polysaccharides, are inulin and numerous fructo- and galacto-oligosaccharides that specifically induce the growth of beneficial *Bifidobacteria* and *Lactobacillus* (reviewed in Roberfroid *et al.* 2010, Parnell & Reimer 2012a). However, currently it seems that actually many more gut bacteria than above-mentioned are affected by the prebiotic consumption thus holistic effects on GM have been reported (Parnell &Reimer 2012b, Everard *et al.* 2014). Usually metabolic effects of probiotics such as reduced body adiposity and increased glucose tolerance are mediated through the increased satiety feeling (Cani *et al.* 2005, Parnell &Reimer 2012b). In addition, prebiotic administration seems to improve the mucosal integrity in gut thus alleviating SLGI (Cani *et al.* 2008). Kellow *et al.* summarized 2014 in a systematic review the scientific evidence supporting the beneficial effects of prebiotics on metabolic health in humans. Twenty-six randomised controlled trials published during 2000-2013 were chosen in the meta-analysis. It seemed that the feelings of self-reported satiety were increased in healthy adults as well as the glucose and insulin levels were reduced in adult subjects as a result of the prebiotic administration (Kellow *et al.* 2014). However, no convergent conclusions could be made of all the other biochemical markers of metabolic health such as weight loss, insulin sensitivity, peptide YY, GLP-1, lipids and inflammatory markers (Kellow *et al.* 2014). As in probiotics further large-scale human randomized, controlled trials are required.

Fecal microbiota transplant (FMT) has been used for some time now in the treatment of recurrent diarrhea caused by *C. difficile* (Kassam *et al.* 2013). As metabolic phenotypes have been successfully transferred via cecal transplant to GF animals, it is possible that the FMT could be an effective way to improve metabolic health and adiposity in humans as well (Turnbaugh *et al.* 2006).

Table 3. Bacterial therapies that have succeeded in the improvement of metabolic diseases in humans based on recent studies.

	DESCRIPTION	EXAMPLES FROM SELECTED HUMAN STUDIES	REFS
Probiotics	Viable microorganisms (bacteria & yeasts) that are believed to provide health benefits to the host when consumed in adequate amounts. (WHO, Park & Bae 2015)	Some *Bifidobacterium* and *Lactobacilli* strains possess hypocholesterolemic effects and improve overall serum lipid profiles in humans.	Reviewed in Ooi & Liong 2010
		L. gasseri decreases adiposity and improves lipid metabolism in overweight subjects.	Kadooka *et al.* 2013, Ogawa *et al.* 2014
		L. gasseri associates with the weight loss in obese subjects	Million *et al.* 2012
		B. animalis subsp. lactis suppresses the translocation of the bacterial components from gut to blood.	Amar *et al.* 2011
		Specific *L. rhamnosus* strain aided the weight loss in obese men and women.	Sanchez *et al.* 2014.
Prebiotics	Non-digestible polysaccharides that stimulate the growth or activity of specific microorganisms (especially *Bifidobacterium* & *Lactobacillus*) in the gut that confers beneficially to well-being of the host. (Gibson & Roberfroid 1995, Roberfroid *et al.* 2010)	Dietary fibre promoted satiety in healthy subjects.	Cani *et al.* 2006
		Oligofructose admistration affected beneficially satiety in overweight adult subjects).	Parnell *et al.* 2009
		Inulin-type fructans selectively affected the GM composition and changed host metabolism modestly in obese women.	Dewulf *et al.* 2013
Fecal Microbiota Transplant (FMT)	The introduction of gut bacteria from a healthy donor into a patient, through transfer of an infusion of a faecal suspension via nasogastric or nasoduodenal tube, rectal enema or the biopsy channel of a colonoscope. (Floch 2010, Lankelma *et al.* 2015)	FMT from lean donors to recipients with MetS induced increased insulin sensitivity and GM diversity.	Vrieze *et al.* 2012

3. AIMS OF THE STUDY

The aim of this study was to evaluate the associations between the overall gut microbiota composition (GMC) and weight and metabolic health of the host in the Finnish study population, and to assess the underlying mechanisms linking GM to obesity and metabolic disorders *in vivo* and *in vitro* in experimental design that involved both cultured adipocytes and hepatocytes.

Specifically, the study aimed to:

1. compare the GMC of metabolically healthy lean and overweight/obese subjects to their counterparts with metabolic disorders (MetS and fatty liver) in order to identify the features of GM that are specific for the metabolic disorders (I-III).

2. evaluate in humans the associations between the molecular signaling pathways in the adipose tissue and GMC in order to further understand the link between adiposity, impaired metabolism and dysfunctional GM (II, III)

3. explore the existing underlying mechanisms that link the adiposity and impaired metabolism to GMC. Specifically, experimental design aimed to reveal:

    a. how flagellin-recognizing TLR5 affect adipose tissue metabolism (III,IV)

    b. through which pathways the GM-derived stimuli affect hepatocyte function (IV).

4. MATERIALS AND METHODS

The thesis work has been a close collaboration project between the Department of Health Sciences at the University of Jyväskylä and Department of Medical Microbiology and Immunology at the University of Turku. Experimental studies were performed in the above-mentioned institutions during the years 2009 – 2015. Thesis work consist of four original publications (I-IV) in which the materials and methods are described in more detail.

4.1 Human study design

The adult participants were recruited from the two larger human cohort studies conducted at the University of Jyväskylä between the years 2008 and 2010. Both studies, namely the AMB study (The role of adiposity-related low-grade inflammation on interactions between adipose tissue, muscle, and bone, The Finnish Academy SKIDI-KID program) and EWI study (Exercise and weight control intervention to study aerobic exercise intervention for improving physical fitness and weight control in overweight and obese women, ISRCTN87529813, Wiklund *et al*. 2014) were performed in accordance with the Helsinki Declaration and were approved by the ethical committee of the Central Finland Health Care district. Signed informed consent was received from all the subjects prior to the assessments. Background information including health status, medical history and lifestyle factors was collected via self-administered questionnaires and a study physician assessed the current health status of the participants. Exclusion criteria in both cohorts were serious cardiovascular, hypertensive, musculoskeletal problems or major liver diseases (assessed by study physician) or diagnosed T1D or other autoimmune diseases. Study design of the thesis is briefly summarized in Figure 7.

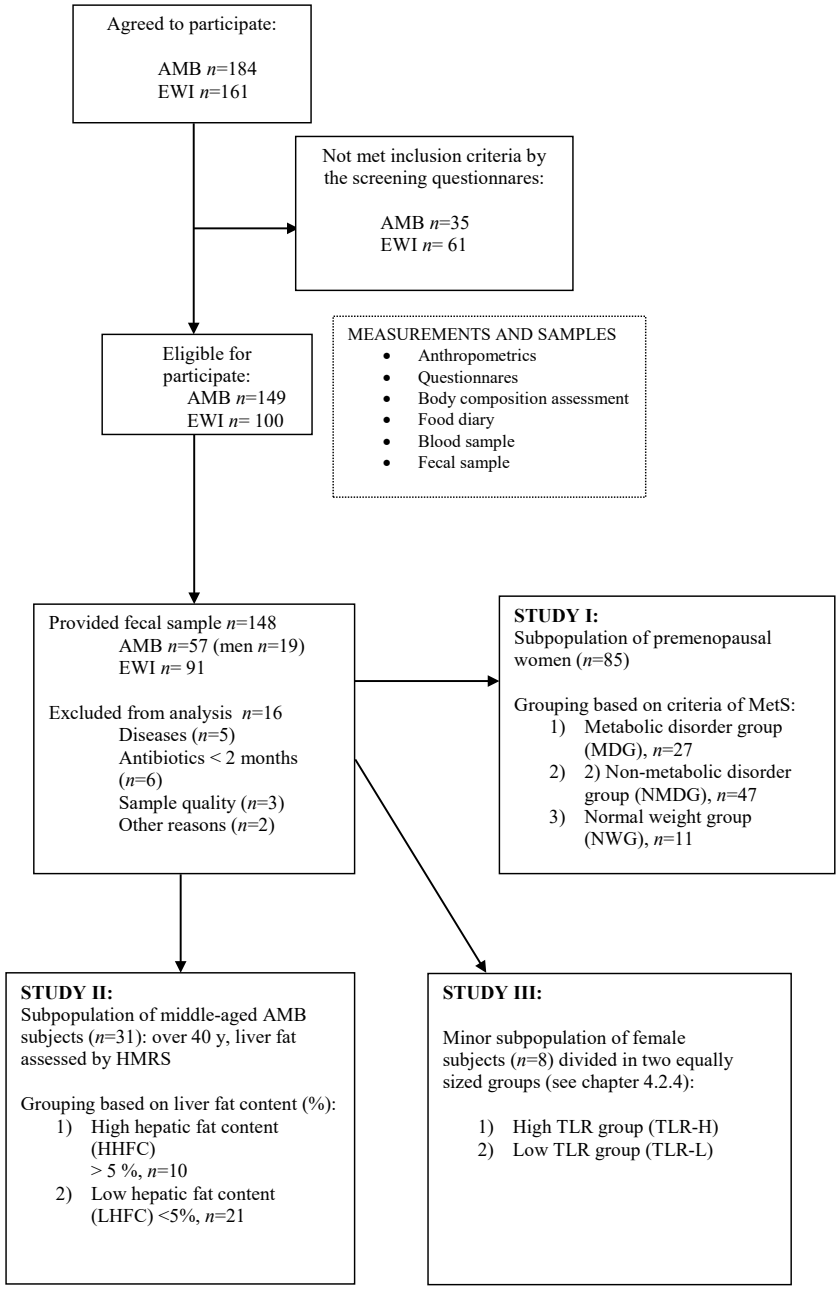

Figure 7. Flow chart of the human study.

4.1.1 Characteristics of human study subjects

Studies I, III and IV included only female subjects in order to exclude the confounding effect of the gender. In Study I from the 85 premenopausal subjects who fulfilled the basic inclusion criteria, 74 were considered as overweight or obese, i.e., had BMI > 25 kg/m^2

and 11 were normal weight, physically active women (NWG). In addition, 27 overweight/obese women fulfilled the criteria of MetS, i.e., expressed the presence of at least three of the following five criteria (Alberti *et al.* 2005): waist circumference (WC) ≥88 cm, resting BP≥130/85 mmHg, fasting serum TG ≥1.7 mmol/L, HDL cholesterol <1.30 mmol/L and glucose ≥5.6 mmol/L and were referred as metabolic disorder group (Figure 7).

Study II included both middle-aged (42-63 years old) men (n = 15) and women (n =16) that were divided into two groups based on their hepatic fat content (HFC, %) that was measured by proton magnetic resonance spectroscopy (Figure 7). The cut-off value for HFC was set to 5 % (Dyson *et al.* 2013). None of the subjects were consumers of excessive amounts of alcohol, i.e., women consumed alcohol <20 g/day and men <30 g/day.

Both study III (n=8) and IV (n=23) included subpopulations of female participants from the AMB study that all provided an adipose tissue biopsy and underwent proton magnetic resonance spectroscopy (^{1}H MRS) assesment. The basis for the group division in Study III is explained further in chapter 4.2.4 and original publication (III). Nor groupings or fecal sample collection were performed in Study IV and the whole study population was pooled for the statistical analysis.

4.1.2 Anthropometrics, body composition and diet (I-IV)

Body height (cm) was measured by using a wall-fixed measuring device, and weight (kg) was determined using an electronic scale, which was calibrated before each measurement session. Body mass index (BMI) was calculated as weight (kg) per height $(m)^2$. Waist circumference was measured twice with a tape measure and the mean value was used (Völgyi *et al.* 2011). Study I utilized bioimpedance assessment (InBody 720, Biospace co., ltd. Korea) in the estimation of body composition i.e. fat mass (FM), FM percentage (FM%), lean mass (LM) and bone mass. Precision of the repeated two measurements expressed as coefficient of variation (CV) was, on average, 0.6 % for FM% (Völgyi *et al.* 2011). Whole body fat mass (FM) was assessed by a Dual-energy X-ray absorptiometry (DXA Prodigy, GE Lunar Corp., Madison, WI USA) in human studies II and III. Two repeated measurements of FM showed a coefficient of variation (CV) of 2.2% (Cheng *et al.* 2009). The assessment of HFC (%) in studies II and IV was performed with ^{1}H MRS (1.5T GE Signa CV/i scanner, GE Medical Systems, Waukesha, WI, USA) by a specialized physician as described in detail before (Borra *et al.* 2009). Spectra were analyzed using the Linear Combination of Model spectra software package (LCModel version 6.1-4, LCMgui user interface version 2.1-4), which is considered as the golden standard for *in-vivo* spectroscopy analysis (Shen *et al.* 2004**).** Dietary information was obtained from a food-intake diary kept for 3 days (two week and one weekend day). The detailed analysis of intakes of energy and energy-yielding nutrients has been described earlier **(**Lyytikäinen *et al.* 2005). Briefly, Micro-Nutrica software (version 2.5) was used in the analysis.

4.1.3 Blood samples (I-IV)

Venous blood samples were taken in the morning (7 to 9 am) after an overnight fast. Serum and plasma were separated and stored at -80 ºC until analyzed further. Plasma glucose and serum TGs, total cholesterol, high-density lipoprotein (HDL) cholesterol and activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analysed using KONELAB 20XTi analyzer (Thermo Fischer Scientific inc. Waltham, MA, USA). Insulin was determined using IMMULITE Analyser (Diagnostic Products Corporation, Los Angeles). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation.

Study IV included a 75-g oral glucose tolerance test (OGTT), and whole-body insulin sensitivity was calculated from glucose and insulin values during the OGTT as previously proposed (Matsuda and DeFronzo, 1999). The inter- and intra-assay CVs were 2.0% and 3.7% for glucose and 11% and 3.4% for insulin, respectively. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as (insulin concentration x glucose concentration)/22.5. Serum leptin was assessed using ELISA (DuoSet®, R&D Systems, MN, USA). Plasma LPS concentration was detected by limulus assay (LAL Chromogenic Endpoint Assay, HyCult biotech, PA, USA).

4.1.4 Gut microbiota profiling of fecal samples (I-III)

Fecal samples were frozen and stored at -80 °C as soon as possible after collection. GM was profiled with a previously described, patented method using 16S rRNA hybridization, DNA-staining and flow cytometry (FCM-FISH, Vaahtovuo *et al.* 2005, Vaahtovuo *et al.* 2008). Six 16S rRNA-targeted oligonucleotide probes that were used in the original studies are presented in Table 4. All probes were labeled at the 5′-end with Cy5 indocarbocyanin (Ex/Em 6467662 nm, Molecular Probes). Samples were analysed as reported previously by using Becton Dickinson FACSCalibur™, Becton Dickinson, San Jose, CA, USA (Vaahtovuo *et al.* 2005, 2008).

Table 4. Targeted oligonucleotide probes used in this study.

PROBE NAME	PHYLOGENETIC TARGET	REF FURTHER IN TEXT AS	REFS
Ato291	*Atopobium* cluster	*Atopobium*	Harmsen *et al.* 2000
Bacto1080	*Bacteroides-Prevotella-Porphyromonas* group	Bacto group	Dore *et al.* 1998
Bif164	Genus *Bifidobacterium*	*Bifidobacterium*	Langendijk *et al.* 1995
Enter1432	γ-*Proteobacteria*	Enter group	Vaahtovuo *et al.* 2005
Erec482	*Clostridium* cluster XIVa	Erec group	Franks *et al.* 1998
Fprau645	*Faecalibacterium prausnitzii*	*F. prausnitzii*	Suau *et al.* 2001

Besides using the abundances of all the different bacterial groups in further analysis, five key figures, i.e., indexes, were calculated from the FCM-FISH data. All indexes were counted individually for each sample, and their purpose was to summarize those bacterial groups in one figure that expressed highest differencies in statistical analysis between the study groups. In Study I the ratio of the Erec group to the Bacto group was calculated (Erec-to-Bacto ratio) by dividing the proportion of the Erec group by the proportion of the Bacto group. In addition, Bacterial index, the sum of proportions of *Bifidobacterium* spp. and the Erec group, divided by the proportion of the Bacto group, was calculated. In Study II *F.prausnitzii* to Bacto group (Fprau-to-Bacto) ratio was calculated by dividing the proportion of *F.prausnitzii* by the proportion of the Bacto group. In addition, previously described Microbial Balance Index (MBI) standing for the overall balance of GM was calculated (Vaahtovuo *et al.* 2007). Due to fact that MBI was originally developed for the description of the GMC in production animals, also MBI2 was further calculated as the sum of abundance of *Bifidobacterium* and *F. prausnitzii*, divided by the sum of abundances of the Erec group and Enter group bacteria. In all studies a rough estimation of the *Firmicutes*/*Bacteroidetes* (F/B) ratio was obtained by adding together the relative proportions of all the analyzed bacterial groups that belonged to the *Firmicutes* (namely the Erec group and *F. prausnitzii*) after which the sum was divided by the proportion of the Bacto group.

4.1.5 Experiments performed with the adipose tissue biopsies (II-IV)

The biopsies from adipose tissue were taken in the morning (7 to 9 am) after overnight fasting under local anaesthesia namely lidokine after skin cooling and disinfection. A needle biopsy (14 G needle, 2.1 ø 60 mm) of subcutaneous abdominal adipose tissue was taken at the level of the navel. The samples were cleaned of any visible connective tissue and blood. Biopsies were frozen in liquid nitrogen after withdrawing from the needle and stored at -80 °C until RNA isolation was performed.

4.1.5.1 Microarray experiments

For host gene expression analysis, total RNA was extracted from the adipose tissue biopsies as described in the original studies (II-IV). Briefly, the FastPrep system and RNeasy Lipid Tissue Mini Kit according to manufacturer's instructions (MP Biomedicals, France, QIAGEN, Gaithersburg, MD, USA) were used. The quality of the total RNA was studied using 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and Experion Automated Electrophoresis Station (BioRad, Hercules, CA, USA). The total RNA was amplified and processed using GeneChip 3´IVT Express Kit (Affymetrix, Santa Clara, CA, USA) and hybridized on Affymetrix Human Genome U219 Array Plates. Microarray results were confirmed by assessing *MMP9* and *VAV1* from the same RNA samples by real-time qPCR. The primer sequences and PCR procedures used in the verification are

presented in the original papers (II-IV). Relative expression levels for *MMP9* and *VAV1* were calculated with the $\Delta\Delta C_t$ method and normalized to the expression of *GAPDH*. The fold changes of each gene between the groups were similar to those detected in the microarray analysis (data not shown). All array data produced during this study has been submitted to ArrayExpress.

4.1.6 Cell lines and cell culture experiments (III, IV)

The human adipose tissue cell line used in studies III and IV originated from Simpson-Golabi-Behmel syndrome patient (SGBS, Fischer-Posovszky *et al.* 2008). The preadipocytes were maintained in the DMEM/F12 media supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 0.33 µM biotin (Sigma-Aldrich, St Louis, USA), 0.17 µM panthotenat (Sigma Aldrich) and penicillin/streptomycin solution (Invitrogen). Prior to the LPS and flagellin (FLG) exposure studies, the preadipocytes were differentiated into the mature adipocytes as described in the original publications (III and IV). Briefly, cells were first incubated for four days in the DMEM/F12 medium that was supplemented with 0.33 µM biotin, 0.17 µM panthotenat, 0.01 mg/ml transferrin, 20 nM insulin, 100 nM cortisol, 0.2 nM triiodothyronine, 25 nM dexamethasone, 250 µM IBMX and 2 µM rosiglitazone (all from Sigma-Aldrich). Further on mature adipocytes were maintained and incubated in DMEM/F12 supplemented with 0.33 µM biotin, 0.17 µM panthotenat, 0.01 mg/ml transferrin, 20 nM insulin, 100 nM cortisol and 0.2 nM triiodothyronine either for 1 or 10 days. Cells were treated either with *E. coli* LPS (100 ng/ml) or *S. typhimurium* recombinant FLG (10 ng/ml) from Sigma Aldrich and Invivogen, respectively, and the effect was compared to to mock-treated control cells.

The HepG2 human liver cells (The American Type Culture Collection ATCC, Rockville, MD) were kindly provided by Dr. Paula Tarkki (University of Jyväskylä). Cells were maintained in the DMEM/Glutamax media supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1% sodium pyruvate (all from Invitrogen, Carslbad, CA, USA). HepG2 cells were directly exposed to recombinant *S. typhimurium* FLG (10 ng/ml). LPS treatment (100 ng/ml) served as the positive control in studies. Upon stimulation the FBS was omitted from the media. Measurement points were 1 h, 4 h and 24 h after treatments. Further, in Study IV HepG2 cells were exposed to the conditioned SGBS culture media (CCM). Briefly, 14-days differentiated adipocytes in 3FC MEDIUM were treated for 24 hours with LPS (100 ng/ml) or FLG (10 ng/ml). Afterwards the culture media was collected and centrifuged for 5 min at 300xg. The freshly collected CCM was then applied to HepG2 cells that were previously washed twice with PBS (Invitrogen).

4.1.6.1 Imaging of the adipocytes by immunofluorescence (III,IV)

For the confocal microscopy imaging, SGBS cells were grown and allowed to differentiate on the cover slips. Prior to the staining they were fixed for 15 min with 4% PFA-PBS, permeabilized with 0.5% Triton-X for 5 min, and blocked after one hour with 5% donkey serum and thereafter incubated with the primary antibody o/n at +4 °C. Immunolabeling was performed using either rabbit polyclonal antibodies against TLR5 (Pierce, Appleton, WI, USA, 1:50 in 1% donkey serum) alone or together with mouse polyclonal antibody against perilipin (Progen, Heidelberg, Germany, 1:200 in 1% donkey serum). Donkey anti-rabbit Alexa Fluor 555, donkey anti-rabbit 488 and donkey anti-mouse Alexa Fluor 555 were used as the secondary antibodies (Invitrogen). The labeled cells were imaged using an inverted wide-field microscope with a confocal unit and 40× oil/1.4 NA objective (both by Carl Zeiss).

4.1.6.2 Western blot analysis

Proteins from both the five-days differentiated adipocytes and hepatocytes were extracted and Western blot analysis was performed as described in the original publications (III and IV). For the Western blot analysis, the protein extracts from the white blood cells were used as the positive control. Finally, the blots were scanned and quantified by using Odyssey CLX Infrared Imager of Li-COR and the manufacturer's software. If re-probing was needed, the membranes were incubated in 0.2 M NaOH for 10 min at RT, washed with TBS and re-probed with appropriate antibodies. All samples and results were normalized to GAPDH.

4.1.6.3 RT qPCR

Cells were homogenized in Trizol reagent (Invitrogen) and the total RNA was extracted according to the supplier's protocol from five-day differentiated adipocytes and HepG2 cells. One to two micrograms of total RNA was reverse transcribed according to the manufacturer's instructions using High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR analysis was performed using in-house designed primers as described in detail in original publications III & IV using iQ SYBR Supermix and CFX96™ Real-time PCR Detection System (Bio-Rad Laboratories, Richmond, CA, USA). Real-time PCR analysis was performed according to MIQE guidelines. The primer sequences, annealing temperatures and PCR conditions are summarized in the original paper (III). Each sample was analyzed in duplicate. Relative expression levels for each gene were calculated with the $\Delta\Delta C_t$ method and normalized to the expression of *ACTB* or *GAPDH*. The amplification efficiencies (E) for each gene were 100±2%.

4.1.6.4 Neutral lipid, Reactive Oxygen Species (ROS) and glycerol measurements in hepatocytes

The Oil Red O stain (Sigma-Aldrich) was used in the measurements of the total amount of neutral lipids and TGs in hepatocytes. Cells were maintained on the 12-well culture plates, and exposed for 24 h either directly to FLG and LPS, or CCM from the treated adipocytes. After the exposure, cells were fixed with 10 % formalin, and then washed with H_2O and 60% isopropanol, and further stained with Oil Red O staining solution at RT for 10 min. Before eluting the staining solution with 100% isopropanol and measuring the absorbance at 550 nm, the cells were washed several times with H_2O. For ROS measurement the cells were treated with FLG, LPS or with the CCM from treated adipocytes for 24 hours. ROS were measured by luminescence using non-lytic ROS-Glo™ H2O2 Assay and Glomax Multidetection System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

For glycerol measurement SBGS adipocytes were allowed to differentiate for 14 days after which the cells were stimulated with FLG for 4 hours. Afterwards the cell culture media was collected and centrifuged for 5 min at 300xg and glycerol was measured using the KONELAB 20XTi analyzer (Thermo Fischer Scientific inc.).

4.1.7 Statistics

For all the original publications, the data was checked for normality using the Shapiro-Wilk's W-test (PASW Statistics 18 or 19). If the data was not normally distributed natural logarithms were used in further analysis. Descriptive data in human studies as well as blood and microbiota parameters are given as means and SD (I, II) or as means with 95 % confidence interval (III, IV). In all analyses statistical significance was set at $p<0.05$.

Study I utilized the analysis of variance (ANOVA) with Fishers Least Significant Difference (LSD) post-hoc test in the comparison of the differences between the three study groups. In addition, further analysis of GMC was performed with the analysis of covariance (ANCOVA). Age and body weight were considered as covariates in the analysis in order to evaluate solely the impact of MetS status on fecal microbiota. Partial Spearman correlation coefficient was used to determine the correlations between variables. Study II utilized Student's paired *t* test in the comparison of study groups and further ANCOVA and the partial Spearman correlation controlled for age, gender and body weight. In Study III Kruskall-Wallis or one-way ANOVA followed with Tukey HSD posthoc test were used to locate the differences between groups in the cell culture experiments and in Study IV ANOVA followed with Bonferroni post hoc tests were performed. Spearman correlation analyses were performed in order to determine the relationship between adipose tissue gene expression and the clinical characteristics. Again the level of significance was set at $p<0.05$.

In studies II and III, the Robust Multiarray Averaging (RMA) algorithm implemented in R package *affy* (Bioconductor) was utilized to normalize and analyze adipose tissue gene expression measurements and differentially expressed genes were detected with the *Limma* R package utilizing linear modeling and empirical Bayes methods (Ihaka & Gentleman 1996, Bolstad *et al.* 2003, Gentleman *et al.* 2004, Gautier *et al.* 2004, Smyth *et al.* 2005). Raw p-values were adjusted using Benjamini and Hochberg multiple adjustment method. Genes with an adjusted p-value below 0.05 were considered as differentially expressed (Benjamini & Yekutieli 2001). The enriched Gene Ontology (GO) terms or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for a given gene set were calculated by utilizing the R packages *GOStat, KEGG.db* and *org.Hs.eg.db*, in which the hypergeometric distribution is used to obtain enriched ontologies and pathways. In the enrichment analysis, all human ENSEMBL genes were used as a background gene group and the categories with a *p*-value lower than 0.05 are considered significantly enriched.

4.2 Results and discussion

The characteristics of the participants in different study groups (**Figure 7**) are given in original papers (I-IV).

4.2.1 Overall description of the GMC

In studies I-III altogether 148 subjects (men n = 19) provided both blood and fecal sample (Figure **7**). The overall, average GMC assessed from the fecal samples that met the inclusion and quality criteria (n = 132) is summarized in Figure 8. The FCM-FISH technique was capable of detecting group-related differences in original studies (I-III). Thus, it seemed to be feasible methodology in the acquisition of the human GMC as has been suggested previously (Rochet *et al.* 2004, Vaahtovuo *et al.* 2005, Vaahtovuo *et al.* 2008). FISH approach has previously shown positive linear correlation with the other state-in- art methodologies such as phylogenetic microarray (Rajilic-Stojanovic *et al.* 2009).

Of all the bacterial groups studied by the FCM-FISH, gram-positive Erec group was the most abundant, in line with the dominance of *Clostridium* cluster XIVa (*Lachospiraceae*) in studies conducted by sequencing (Eckburg *et al.* 2005, Turnbaugh 2009) and other molecular analyses (Rajilic-Stojanovic *et al.* 2009). Altogether it seemed that the relative abundances of all the groups and genus analysed were concordant with the previously published characterization based on the FCM-FISH methodology utilizing same SSU 16S probes and frozen samples (Nadal *et al.* 2009, De Palma *et al.* 2009, De Palma *et al.* 2010). The abundance of the Bacto group seemed to be quite low compared to the human studies conducted with freshly prepared fecal samples (Rochet *et al.* 2004, Vaahtovuo *et al.* 2008). During the development of the FCM-FISH methodology it was

observed that the freezing of the fecal sample affects especially the Bacto group so that its relative abundance decreases (unpublished results). Similar drop in the abundance of *Bacteroidetes* in the fecal samples after freezing has also been observed after DNA extraction and downstream analysis by qPCR (Bahl *et al*. 2012). Probe additivity was nearly 34 %, which is lower than in human studies conducted with fresh fecal samples utilizing the same oligonucleotide probes (Rochet *et al.* 2004, Vaahtovuo *et al.* 2008). Due to targeted phylogenetic approach, it is possible that some differences in GMC between the healthy and metabolically impaired individuals were not detected in original studies (I-III). However, the handling procedures regarding sampling, preservation and processing of the fecal samples were consistent throughout the study. Thus, it was possible to perform reliable comparisons between the samples.

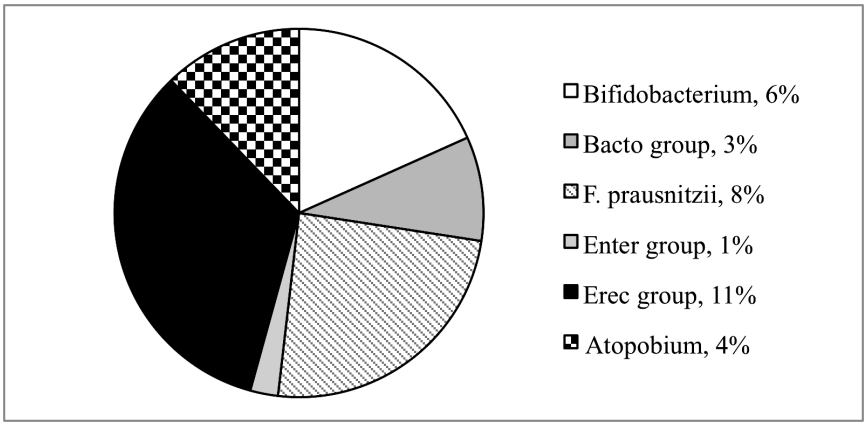

Figure 8. Overall GMC of all the study subjects determined by FCM-FISH a) Average abundances (%) of the six targeted bacterial groups, representing dominant and/or important intestinal bacteria as specified in Table 5. Data is presented as mean.

All the samples were pooled for the preliminary analysis (n=132) and grouped based on gender, age and metabolic health status. Three different age groups were formed for the analysis: young adults (below 30 years), early middle-aged subjects (30-52 years-old) and late middle-aged subjects (over 52 years). The division depending on the metabolic status of the subjects was based on the previously described criteria of MetS (Alberti *et al.* 2005). Despite the large interindividual variation observed in the human samples, significant differences in the GMC were observed after the utilization of different groupings of the samples. In addition, both MBI and MBI2 (average values 7.5 and 1.6, respectively) that are counted from the proportions of four major bacterial groups present in the gut, seemed to serve as a practical approach in the comparison of different groups. Hence, in the future these gut indexes could be useful tools for example in monitoring the changes in GMC in intervention studies that aim to GM modulation.

Preliminary results showed that gender had an effect on the abundance of Enter group, which was greater in women ($p < 0.001$). Women also had higher MBI ($p = 0.016$). The abundance of the Bacto group, *F. prausnitzii* and Enter group increased significantly with the age ($p < 0.05$ for all). In addition, age tended to affect the relative proportion of the Erec group that was highest in the oldest individuals ($p = 0.056$). Thus age and gender were taken into account as confounding factors in the statistical analyses performed in the original publications (I-III). Subjects fulfilling the criteria of MetS expressed a higher abundance of the Erec group bacteria in their feces ($p < 0.001$). In addition, MBI2 was significantly lower in the MetS group compared to the healthy controls ($p = 0.014$).

4.2.2 Subjects with metabolic disorders have a significantly different gut microbiota profile compared to their metabolically healthy counterparts (I)

In Study I, the overweight and obese subjects fulfilling the criteria of MetS had a significantly greater proportional amount of the Erec group, i.e., representatives of the *Clostridium* cluster XIV in their feces than their metabolically healthy counterparts ($p = 0.008$) and normal weight controls (NWG, $p = 0.006$). All the three study groups reported similar energy intake and no other differences regarding the content of diet were observed (data not shown). Interestingly, as the confounding effect of the body weight was taken into account in statistical analysis, a difference remained between the two groups consisting merely of overweight/obese subjects but disappeared when compared to NWG. In addition, subjects with MetS had a higher proportion of potentially inflammatory gram-negative Enter group bacteria than NWG ($p = 0.043$). Thus, these results suggest that a metabolically aberrant GMC may associate with obesity combined with MetS but not obesity *per se*. Interestingly, a recently performed metagenomic study observed that not all obese individuals possess the impaired GMC described as decreased microbial richness (Le Chatelier *et al.* 2013). Thus, it seems reasonable that there is a subgroup of individuals that is more prone to the onset of metabolic disorders via the effect of GM.

The Erec group associated significantly with the various traits of MetS (Table 5). Further, analysis of the whole study population revealed that the greater amount of the Erec group in the fecal microbiota positively correlated with greater body weight, BMI, fat mass, FM% and visceral fat area ($p < 0.01$ in all, Table 5), whereas gram-negative Bacto group inversely correlated with the above-mentioned factors ($p < 0.05$ in all Table **5**). In addition, Erec positively correlated with serum TG levels and negatively with HDL cholesterol concentrations (both $p < 0.05$, Table 5). On the contrary, Bacto group correlated positively with HDL cholesterol but negatively with insulin (both $p < 0.01$, Table 5). The observed associations were stronger when the individual Erec-to-Bacto ratios were utilized in the analysis emphasizing the importance of analysis of not just singular bacterial species or groups but entities.

Table 5. All the statistically significant correlations between GMC and traits of MetS in Study I.

VARIABLES	EREC GROUP	BACTO GROUP	EREC-TO-BACTO
Weight (kg)	++	−−	+++
BMI (kg/m^2)	+++	−	+++
Fat mass (kg)	++	−	+++
Fat mass %	++	−	+++
Visceral fat area (cm^2)	++	(−)	+++
Insulin (mU/L)	(+)	--	++
HDL (mmol/L)	−	++	−−−
TG (mmol/L)	++	(−)	+++

+++ = strong, positive correlation, ++ = moderate positive correlation,
+ = weak, positive correlation, (+) positive trend, −−− = strong, negative correlation
−− = moderate, negative correlation, − = weak, negative correlation, (−) negative trend

The Erec group includes a variety of gram-positive bacterial species that are capable of digesting complex dietary carbohydrates such as plant polysaccharides (Turnbaugh *et al.* 2006, Mahowald *et al.* 2009). These bacteria are rather stable members in the gut at least in short-term, but notable inter-individual variation both in the abundance and species diversity has been reported (Maukonen *et al.* 2012). Increased carbohydrate metabolism via microbial fermentation stemming from the *Firmicutes*-rich microbiome has been shown in experimental models to enhance the monosaccharide absorption from the gut and further stimulate increased lipogenesis and triglyceride storage in adipocytes (Turnbaugh *et al.* 2006). Recently corroborative results about the high abundance of Erec group bacteria in obesity and MetS have been shown in various human studies (Ferrer *et al.* 2013, Tims *et al.* 2013, Verdam *et al.* 2013). In addition, a recent rodent study showed that the obese-prone and dysmetabolic rats had a higher abundance of several Erec group bacteria than their obese-resistant counterparts after a 12-week intervention on HFD (Duca *et al.* 2014).

In addition, a phylum-based microbial marker, namely *Firmicutes*/*Bacteroidetes* (F/B) ratio, was calculated from the existing bacterial data. High F/B ratio has been associated with the obese phenotype both in mice and humans based on the early landmark studies (Ley *et al.* 2005, Turnbaugh *et al.* 2006, Ley *et al.* 2006, Turnbaugh *et al.* 2009). The F/B ratio was higher in MDG compared to the so-called healthy obese and NWG but after adjustment for weight there were no differences among the groups ($p > 0.05$ for all, data not shown). Thus, this finding raises a question about the usefulness of F/B ratio in the studies concerning GM in metabolic disorders as concordantly has been proposed also by others (Arumugam *et al.* 2011, Walters *et al.* 2014). However, it should be kept in mind that in this study the F/B ratio was just a rough estimate since due to targeted FCM-FISH analysis approach nearly not all members from both the *Firmicutes* and *Bacteroidetes* phylum were actually detected.

4.2.3 Aberrant gut microbiota associates with higher liver fat content in human subjects simultaneously with adipose tissue inflammation (II)

In Study II, human subjects whose HFC exceeded 5 % had less *F.prausnitzii* in their feces compared to those with liver fat content less than 5 % ($p = 0.047$). The difference in the *F.prausnitzii* abundance between the two groups remained significant after adjusting for age, gender and weight ($p = 0.030$). In addition, the HHFC group tended to have a higher amount of gram-negative, potentially pro-inflammatory Enter group bacteria in their feces but this difference was non-significant due to the high inter-individual variance observed (aver. 1.4 % vs. 0.5%). The lower abundance of anti-inflammatory *F. prausnitzii* with simultaneously observed higher abundance of potentially pro-inflammatory Enter group bacteria might indicate that GM dysbiosis prevail in the HHFC group. Several Enter group bacteria contain LPS structure and locomotive organelle flagella that both have been linked to the increased systemic inflammation via activation of TLR4 and TLR5 signaling pathways that can also contribute to the hepatic fat accumulation (Cani *et al.* 2007a, Vijay-Kumar *et al.* 2010).

These results provide the novel evidence regarding a link between the low abundance of *F. prausnitzii* and high hepatic fat accumulation in humans. *F. prausnitzii* has emerged as a key bacterium in the maintenance of gut homeostasis and high levels are considered to protect the host from various diseases such as obesity, MetS and NAFLD (Le Chatelier *et al.* 2013, Walters *et al.* 2014, Remely *et al.* 2015a, Stenman *et al.* 2015). The depletion in the *F. prausnitzii* abundance has been previously linked to IBD and colitis (Sokol *et al.* 2008, Sokol *et al.* 2009). Interestingly, *F. prausnitzii* abundance increases as a result of fasting, thus low energy intake seems to favor this bacterium (Remely *et al.* 2015b). *F. prausnitzii* possesses anti-inflammatory properties *in vitro* and thus may be able to improve the gut barrier integrity and further alleviate the state of systematic metabolic endotoxemia caused by GM dysbiosis (Sokol *et al.* 2008, Carlsson *et al.* 2013). Therefore, it is likely that the decreased amount of *F. prausnitzii* may lead to the increased gut permeability and the leakage of inflammatory factors, such as LPS and further to SLGI and IR in other tissues.

Besides *F. prausnitzii*, significant differences between the study groups were also observed in the two bacterial indexes that reflect the ratio of different bacterial groups that has been previously implicated in metabolic diseases. Both FPrau-to-Bacto ratio and MBI were lower in the HHFC group ($p = 0.004$ and 0.024) and differences remained significant after adjusting for gender, age, and weight ($p = 0.003$ and 0.011, respectively). When all the subjects were analysed together, *F. prausnitzii*, Fprau-to-Bacto ratio, and MBI all correlated negatively with HFC % ($p < 0.05$ for all) and importantly Enter group tended to correlate positively with HFC % ($p = 0.079$). After adjustment for age, gender and weight, the associations of *F. prausnitzii*, Fprau-to-Bacto, and MBI with HFC %

remained significant (r = -0.446, p = 0.049; r = -0.639, p = 0.002 and r = -0.724, p <0.001, respectively). Fprau-to-Bacto ratio and MBI negatively correlated with HOMA-IR (r = -0.678, p = 0.001 and r = -0.614, p = 0.004). In addition, MBI negatively (r = -0.450, p = 0.046) correlated with TG levels. The F/B ratio tended to be lower in the HHFC group (p = 0.067) but became significant after adjustment for gender, age and weight (p = 0.019).

Even though no significant differences in the amounts of the Bacto group between the high and low HFC groups were observed, a positive association existed between this gram-negative group and HOMA-IR after the adjustment for gender, age, and weight (r = 0.478, p = 0.033). A recent study contradictorily reported that patients with diagnosed NASH harbored lower levels of *Bacteroidetes* in their gut than healthy controls (Mouzaki *et al.* 2013). However, this assessment was done by utilizing a different methodology, namely qPCR, and utilized diagnosed patient groups. Hence, these factors may account for the differences between the results. In addition, a different statistical analysis approach was utilized since the above-mentioned results were not controlled for age, weight, and gender. In addition, recent systematic review (five human studies) concluded that no unambiguous phylogenetic markers (especially related to *Bacteroidetes/Bacteroides*) describe the GMC in a state of fatty liver and urged especially longitudinal studies (Wieland *et al.* 2015).

Finally, gram-negative Enter group associated positively both with HFC and serum TG levels (r = 0.634, p = 0.002 and r = 0.634, p = 0.002), but despite these significant positive correlations, no differences were observed neither in plasma LPS levels nor adipose tissue *TLR4* expression between the groups. Several animal studies have shown that LPS can induce adipose tissue inflammation and increase serum FFA and TG *via* TLR4 (Cani *et al.* 2007a). However, our study did not include the medically verified steatotic patients, which may at least partly explain why metabolic endotoxemia was not observed in the HHFC group.

4.2.3.1 Adipose tissue gene expression in HHFC individuals and its association with GMC

Adipose tissue microarray analysis (presented in Table 3 in the original publication II) revealed that the HHFC group expressed differentially genes (n = 27) that relate to the innate and adaptive immunity and to the macrophage infiltration (adjusted p <0.05). The most up-regulated transcripts in the HHFC group were *CHI3L1* and *MMP9* (p = 0.019 and 0.024 respectively) and only one gene, *MAPK10*, was down-regulated (p = 0.038). No significant fold differences were observed in the expression of several lipid metabolism-related genes (e.g. *ACAT1, LYPLA2, ACACA, ACSL5, HADH, CHAT, CRLS1* etc.) between the groups. In addition, no statistically different gene expressions

between lean subjects with HHFC and obese with LHFC were observed (data not shown), suggesting that the gene expression in subcutaneous abdominal fat is not driven alone by the amount of liver fat and metabolic health status but also affected by adiposity. Further, the comparison of the gene expression values between the lean and the obese within the HHFC group revealed no differences either (data not shown), which suggests that it is not the obesity *per se* that causes the liver fat accumulation (Kotronen & Yki-Järvinen 2008).

All the differentially up-regulated genes, such as *DOCK2, MMP9* and *RAB34,* correlated positively with HFC % (see original paper II). In addition, FPrau-to-Bacto ratio correlated negatively with the expression level of *CHI3L1* ($r = -0.522$, $p = 0.046$), *CD52* ($r = -0.615$, $p = 0.015$), *DOCK2* ($r = -0.626$, $p = 0.013$), *LAPTM5* ($r = -0.562$, $p = 0.029$), *MMP9* ($r = -0.586$, $p = 0.022$), *NKX24* ($r = -0.713$, $p = 0.003$), *RAB34* ($r = -0.537$, $p = 0.039$), and positively with *MAPK10* expression ($r = 0.563$, $p = 0.029$). The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that the enriched pathways in HHFC adipose tissue included natural killer cell-mediated cytotoxicity, T cell signaling and leukocyte transendothelial migration, indicating a presence of inflammation in the adipose tissue. Those over-expressed genes in the HHFC adipose tissue that negatively associated with FPrau-to-Bacto ratio play a role for example in the cytoskeleton reorganization, degradation of the extracellular matrix, innate immunity, immune response regulation, cytokine activation, antigen presentation, T cell regulation and microbial pathogen recognition and have been related to bacterial infections (Fukui *et al.* 2001, Watanabe *et al.* 2006, Hosoki *et al.* 2012, Glowacka *et al.* 2012, Paolillo *et al.* 2012, Kasmapour *et al.* 2013). Thus, an inflammatory response against bacteria and/or bacterial components in the adipose tissue may link the GMC to the excess of hepatic fat. Further, the adipose tissue inflammation is known as one of the driving mechanisms in the development of IR and hyperlipidemia, and it may represent a link between GMC and HOMA-IR and serum TGs in this study.

4.2.4 Over-expression of Toll-like receptor 5 in subcutaneous adipose tissue associated with obesity, metabolic changes and dysbiotic gut microbiota composition in humans (III)

An adipose tissue microarray database (Kong *et al.* in preparation) was utilized for the comparison of the expression rates of several genes in the TLR5 signaling pathway (KEGG ID hsa04620) from the subcutaneous adipose tissue samples from women that participated in the AMB study. As to date no reference values for the adipose tissue *TLR5* expression exists, the subjects with extreme values were included in the comparison. Clinical characteristics and GM structure of the subjects

that overexpressed the TLR5 pathway (H-TLR group, *n*=4) were compared to those who had the lowest gene expression levels in their adipose tissue (L-TLR group, *n*=4). The respective fold differences in the TLR5 pathway genes between the study groups were as follows: TLR5 1.9, CD86 1.9, CCL4 4.5, Ly96 2.8, MyD88 1.2 and Jun 2.0, respectively.

Interestingly, the groups did not differ on the expression levels of other TLRs such as 2 and 4 that have previously been linked to obesity and IR. The adipose tissue microarray data analysis revealed 419 up-regulated and 249 down-regulated genes in the H-TLR group compared to L-TLR group (adjusted $p < 0.05$). The enriched pathways in the H-TLR group were related to the metabolism and inflammation, for example B cell signaling and leukocyte transendothelial migration based on the gene set enrichment analysis on KEGG pathways and Gene Ontology terms (Figure 9). The findings of the microarray data were verified by using Gene Expression Omnibus (GEO) and the GDS3679 dataset (MacLaren *et al.* 2010). By utilizing the GEO2R tool the subjects with highest ($n = 3$) and lowest ($n = 3$) *TLR5* expression were compared. Nearly 80% of the differentially expressed genes (DEGs) that were involved in the enriched KEGG pathways (Figure 9) were similarly up- and down-regulated in the GDS3679 dataset, indicating the robustness of the findings.

Several genes involved in the pathways related to the cellular respiration (e.g., *IDH, OXA*, and *UQCR*) were found to be down-regulated and others related to vasculogenesis were over-expressed (e.g., *EGFL6, VEGFA, MMP9*, and *MMP19*) in the H-TLR group designating local hypoxia in the adipose tissue (Supplementary Table 1 in the original publication III). In addition, several genes related to the apoptosis and inflammation-induced cell death (e.g. *BAG3, CASP3, TNFRSF12A*, and *TNFRSF1B*) were over-expressed in the adipose tissue of the H-TLR subjects. This may suggest the occurance of adipocyte hypertrophy which may lead via local hypoxia to the subsequent cell death (Gambero *et al.* 2007).

In addition, genes involved in the complement pathway C1Q were over-expressed in the H-TLR group adipose tissue. C1Q is a classic pathway that is induced during the innate immunity reaction caused by bacterial activation. Interestingly, in the H-TLR adipose tissue mechanisms related to response to the *E. coli* infection were activated as shown by the over-expression (Figure 9). Recently the involvement of pathogenic gram negative tissue bacteria in diabetes and obesity has been shown (Amar *et al.* 2011).

KEGG ID	P value	Count	Size	Term	Gene names in the pathway
280	0.0003	9	46	Valine, leucine, and isoleucine degradation	*ACADM, ALDH6A1, AUH, BCKDHB, DBT, HADH, MUT, PCCA, PCCB*
1032	0.0005	7	31	Glycan structures degradation	*GBA, GLB1, GNS, GUSB, HEXB, HPSE, MAN2B1*
640	0.001	7	35	Propanoate metabolism	*ACACB, ACADM, ACSS3, ALDH6A1, MUT, PCCA, PCCB*
531	0.001	5	18	Glycosaminoglycan degradation	*GLB1, GNS, GUSB, HEXB, HPSE*
4510	0.003	19	203	Focal adhesion	*ACTB, ACTG1, BIRC3, COL6A1, COL6A2, COL6A6, FLNA, GRB2, ITGB5, JUN, MYL9, PDGFA, PIK3R5, PPP1CA, RAC2, SPP1, VAV1, VEGFA, ZYX*
603	0.003	4	14	Glycosphingolipid biosynthesis globo series	*GBGT1, GLA, HEXB, NAGA*
5130	0.004	8	54	Pathogenic *Escherichia coli* infection (EHEC)	*ACTB, ACTG1, CD14, HCLS1, LY96, TUBA1C, TUBB2A, TUBB2B*
5131	0.004	8	54	Pathogenic *Escherichia coli* infection (EPEC)	*ACTB, ACTG1, CD14, HCLS1, LY96, TUBA1C, TUBB2A, TUBB2B*
4670	0.004	13	119	Leukocyte transendothelial migration	*ACTB, ACTG1, CYBA, GNAI1, ICAM1, ITGB2, MMP9, MSN, MYL9, NCF4, PIK3R5, RAC2, VAV1*
4610	0.005	9	69	Complement and coagulation cascades	*C1QA, C1QB, C1QC, C1R, C1S, C3AR1, C6, F13A1, SERPINE1*
4662	0.011	8	65	B cell receptor signaling pathway	*BLNK, FCGR2B, JUN, PIK3R5, PTPN6, RAC2, SYK, VAV1*
530	0.013	5	30	Aminosugars metabolism	*CHIT1, HEXB, HK3, NAGK, NPL*
1040	0.019	4	22	Biosynthesis of unsaturated fatty acids	*ACOT7, FADS1, PECR, PTPLB*
910	0.025	4	24	Nitrogen metabolism	*CA2, CA3, CTH, GLUL*
4650	0.027	12	135	Natural killer cell mediated cytotoxicity	*FCER1G, GRB2, HCST, ICAM1, ITGB2, PIK3R5, PTPN6, RAC2, SYK, TNFSF10, TYROBP, VAV1*
5110	0.027	7	62	Vibrio cholerae infection	*ACTB, ACTG1, ATP6V0B, ATP6V1F, KCNQ1, PRKX, TCIRG1*
52	0.033	4	26	Galactose metabolism	*GAA, GLA, GLB1, HK3*
511	0.038	3	16	*N*-Glycan degradation	*GLB1, HEXB, MAN2B1*
4060	0.044	19	263	Cytokine-cytokine receptor interaction	*CCL13, CCL18, CCL19, CCL2, CCL22, CCL3, CCL4, CSF1R, CSF2RB, CXCL16, GHR, IL10RA, PDGFA, TNFRSF12A, TNFRSF1B, TNFSF10, TNFSF13B, TSLP, VEGFA*
4614	0.044	3	17	Renin-angiotensin system	*AGTR1, ANPEP, CTSG*

Size is the total amount of genes involved in this pathway. Count is the amount of differentially expressed genes that map in this pathway.

Figure 9. KEGG Pathway enrichment of the differentially expressed adipose tissue genes in the H-TLR group. (Pekkala *et al.* 2015)

4.2.4.1 The clinical manifestations, metabolism and GMC in H-TLR group

Despite the small sample size (n = 4/group), the clinical characteristics between the subjects with high and low TLR5 expression pathways differed markedly. The H-TLR group seemed to have more pronounced obese phenotype, which was shown by a higher avegare body weight, and greater WC and total body fat percentage than the L-TLR group ($p < 0.05$ for all). In addition, the average systolic and diastolic BP of H-TLR subjects were above the reference range (130/85 mmHg) and were significantly higher than in the L-TLR group ($p < 0.05$). The H-TLR group were metabolically impaired since the levels of two important hormones in the energy metabolism, namely leptin, accompanied with adiponectin, significantly differed from the L-TLR group (67.0 vs. 21.6 ng/ml and 7.1 vs. 19.6 ng/ml, p <0.05 for both).

The comparisons of the GMC revealed significant differences between the two groups, namely the H-TLR group had a greater fecal abundance of Erec group bacteria (p = 0.029) and also the F/B ratio tended to be higher than in L-TLR (p = 0.057). No other significant differences were found in the bacterial groups. However, this may arise from the small number of subjects and relatively high inter-individual variation. However, compared to the H-TLR group the L-TLR individuals had on average 20 % greater abundance of *Bifidobacterium* spp. that are known to be beneficial members of GM maintaining the integrity of the epithelial barrier in the gut, thus decreased amounts of *Bifidobacteria* spp. might imply increased intestinal permeability (Cani *et al.* 2008).

The above-mentioned results suggest several other possible underlying mechanisms. A simultaneous increase in the *Bifidobacteria* spp. and a decrease in the Erec group bacteria in the gut had beneficial effects on the adipose tissue gene expression in obese mice (Dewulf *et al.* 2011). Some members of the Erec group possess flagella enabling the movement of these bacteria (Neville *et al.* 2013). Gut bacteria that have the ability to move endow a significant immunostimulatory potential since they are potential TLR5 activators. In addition, the representatives of the Erec group are known to induce colonic T_{reg} cells and their abundance has been associated with immunodominance in Crohn's disease, characterized by increased intestinal permeability (Duck *et al.* 2007, Atarashi *et al.* 2011). In addition, in colitis murine model heat-killed, flagellated *E. rectale* strain A1-86 stimulated NF-κB activation through both TLR2 and TLR5 (Erridge *et al.* 2010), and its protein preparation stimulated the secretion of IL-8 from human IECs *in vitro* (Neville *et al.* 2013). Thus, the aberrant gut microbiota described as the increased fecal abundance of the Erec group and decreased *Bifidobacterium* observed in the H-TLR group might increase gut permeability, which together with the elevated abundance of gut-derived flagellar antigens promotes the adipose tissue inflammation through the activation of TLR5. Important notion is that the *S. typhimurium* FLG used in current *in vitro* experiments shares a similar structure with *E. rectale* FLG (Neville *et al.* 2013).

Some Erec group members are also notable SCFA producers (especially butyrate), from which the host usually benefits at normal production range (Flint *et al.* 2012). However, a too high production rate together with the decreased catabolism might induce lipogenic effects on the host (Zambell *et al.* 2003). In the adipose tissue of the H-TLR group several genes related to the SCFA catabolism (e.g., *AACS, ACSS3, HADH, ACACB*, and *ACADM*) were downregulated while the lipogenic genes were over-expressed (Figure 9). Thus, the increased production of SCFA by the Erec group together with the decreased catabolism may create a vicious circle of ongoing lipogenesis and inflammation.

Some of the effects of TLR5 may be mediated by the adipokine leptin that was increased in the circulation of H-TLR subjects. Interestingly, leptin is known for the ability to upregulate the expression of several TLRs in adipocytes (Batra *et al.* 2007). Thus the high levels of leptin might induce the target cells to become resistant to its actions, which may further contribute to the development of obese phenotype and metabolic impairment observed in H-TLR subjects.

4.2.5 In vitro studies in cultured human adipocytes: FLG induces functional changes in the adipocyte metabolism (III)

The presence and expression of the FLG-induced TLR5 in mature human adipocytes (5- and 14-days differentiated) was verified by the Western blot and immunofluorescence

followed imaging by confocal microscopy (Figure 10). White blood cell protein extracts served as controls in Western blot. Interestingly, imaging revealed that in the mature SGBS adipocytes (14 days differentiated) the majority of the TLR5 is located in the different intracellular compartments such as lipid droplets and nuclei (Figure 10B-D) and only a minor pool was located on the cell surface (Figure 10A). There has been quite a scientific debate regarding the possibility of whether TLR5 is located inside the human cells or not. In DCs, TLR5 has been found on the cell surface but for example in the breast cancer cell lines both on the surface and in intracellular compartments (Cai *et al.* 2011, Shibata *et al.* 2012). Our experiments located the majority of TLR5 in the intracellular compartments of adipocytes.

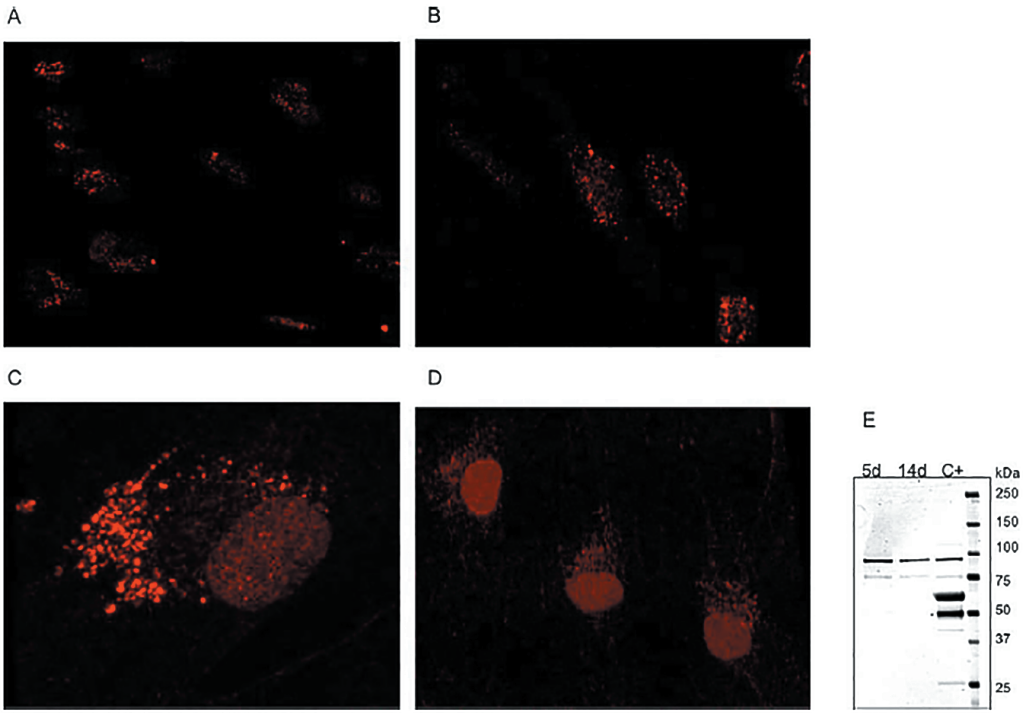

Figure 10. The confocal microscopy images of intracellular localization of TLR5 in human adipocytes. Only a minority of the TLR5 pool is located in membrane structures (A). The majority of the TLR5 pool is intracellular in 14-days (B). A part of the pool seems to be localized in the nuclei and lipid droplet-like structures (C,D). Expression of TLR5 (90 kDa) by Western blot (E, 5-days and 14-days, C+ = control white blood cells). (Pekkala *et al.* 2015)

Further changes in the adipocyte metabolism and function were observed by the 1 h and 4 h FLG-stimulation as summarized in Table 6. Briefly, FLG induced a 3.4-fold increase in the level of *TLR5* in adipocytes ($p = 0.006$) but did not cause any change to the expression of *TLR4*. Further, an increase in the expression of both *NF*-k*B* (1.8-fold, $p = 0.026$) and *SCD1* (1.5-fold, $p = 0.035$) was observed after 1 h treatment. *NF*-

k*B* is a downstream target for TLRs and stearoyl coenzyme desaturase 1 (SCD1) and relates to the lipogenesis. Further, 4 h treatment caused an increase in the expression of the inflammatory matrix metalloprotease 9 gene (*MMP-9)* by 1.9-fold ($p = 0.001$). Interestingly, *MMP-9* was also overexpressed in the H-TLR group adipose tissue as previously mentioned. After the 4 h treatment, the expression of *TLR4* (1.7-fold, $p = 0.015$), *MMP-9* (1.5-fold, $p = 0.003$), and *SCD1* (1.4-fold $p = 0.045$) were increased in response to the control aka the LPS treatment. In addition, increased lipolysis was observed in the 14-days differentiated adipocytes by the effect of both FLG and LPS since both treatments increased glycerol secretion significantly at 4 h ($p < 0.05$ for both). In addition, merely FLG triggered the increased production of inflammatory reactive oxygen species (ROS) after 24 h ($p = 0.04$).

Table 6. The effect of the treatment (FLG or LPS) on mRNA levels in SGBS adipocytes**.**

	FLG, 1 H	**FLG, 4 H**	**LPS, 1 H**	**LPS, 4 H**
TLR5	3.4-fold $p = 0.006$	NS	2.6-fold $p = 0.033$	NS
TLR4	NS	NS	NS	1.7-fold $p = 0.015$
NF-κB	1.8-fold $p = 0.026$	NS	1.7-fold $p = 0.047$	NS
SCD1	1.5-fold $p = 0.035$	NS	1.5-fold $p = 0.009$	1.4-fold $p = 0.045$
MMP-9	NS	1.9-fold $p = 0.001$	NS	1.5-fold $p = 0.003$

MMP-9 = matrix metalloprotease 9, *NF*-k*B* = nuclear factor kappa B, *SCD1* = stearoyl coenzyme desaturase 1, NS = not significant fold-change in expression compared to control

Thus, it can be concluded that the TLR5 activation in the adipose tissue might at least partly be responsible for the metabolic impairment observed in the H-TLR group since our *in vitro* experiments using bacterial FLG as TLR5 agonist, highlight its pro-inflammatory effects, reflected as higher *NF-κB* expression and ROS production. However, the possible role of endogenic TLR5 antagonists cannot be excluded. Moreover, increased *SCD1* expression in the adipocytes was observed. Accordingly, SCD1-deficient adipocytes have been shown to exhibit reduced inflammatory response to LPS treatment (Liu *et al.* 2011). However, even though the *SCD1* expression increased, the level of Fatty acid synthase (FAS) gene did not. It seems that the overall lipogenesis is not enhanced by the FLG stimulation. It is also previously shown that the ROS production activates the inflammatory pathways including NF-κB, as well as the fatty acid desaturation pathway that is mediated by the SCD1, which is in agreement with the results observed in this study (Busch *et al.* 2005). TLRs may play the significant role also in this cellular process (Dasu *et al.* 2012).

4.2.5.1 Insulin signaling studies

The effects of FLG and LPS on insulin signaling and other intracellular, metabolism-related signaling routes were evaluated by assessing the phosphorylation levels of the various key proteins in these routes. Again 5-days differentiated adipocytes were used in the analysis. Both FLG and control LPS decreased the phosphorylation of Akt (p <0.05 for both) but only FLG increased the phosphorylation of the Mammalian target of rapamycin (mTOR) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) ($p < 0.001$ and $p = 0.042$, respectively). In addition, the phosphorylation of eukaryotic initiation factor 4E binding protein (4EB-P1) that resides downstream from the mTOR and AMPK seemed to increase due to both treatments, but did not reach the statistical significance.

TLR5 is known to induce inflammation through the activation of several kinases and the NF-κB pathway (reviewed in Könner & Bruning 2011). FLG induced *NF-κB* expression, ERK1/2 phosphorylation and the inhibitory serine phosphorylation of IRS1 and enhanced the mTOR phosphorylation in SGBS adipocytes accordantly to the various previously reported results (Ozcan *et al.* 2004, Tanti & Jager 2009).

4.2.6 Adipose tissue *TLR5* mRNA correlates with hepatic fat content and insulin sensitivity in humans (IV)

Human adipose tissue *TLR4* and *TLR5* expression levels were determined from the microarray data (IV). There was a strong positive correlation between the *TLR5* expression in the adipose tissue and HFC % ($r = 0.699$, $p = 0.003$) and a strong negative correlation between the insulin sensitivity index ($r = -0.529$, $p = 0.016$) and the adipose tissue *TLR5* expression (Figure 11). Interestingly, no associations between *TLR4* expression levels and either HFC % or insulin sensitivity were observed. This observation is consistent with the findings reported in Study II thus the human subjects with high HFC % did not differ on plasma LPS levels from the subjects with low HFC %. The central role for TLR4 in the onset and progression of NAFLD is well established (Miura *et al.* 2010). Thus the controversial results of this study may suggest that the effects of TLR4 *in vivo* are not universal and depend largely on GMC, gut integrity and/or the bacterial species from which the circulating LPSs are derived. Further, it seems that TLR5 is much relevant biomarker in metabolic disorders than TLR4 at least when subcutaneous adipose tissue is studied.

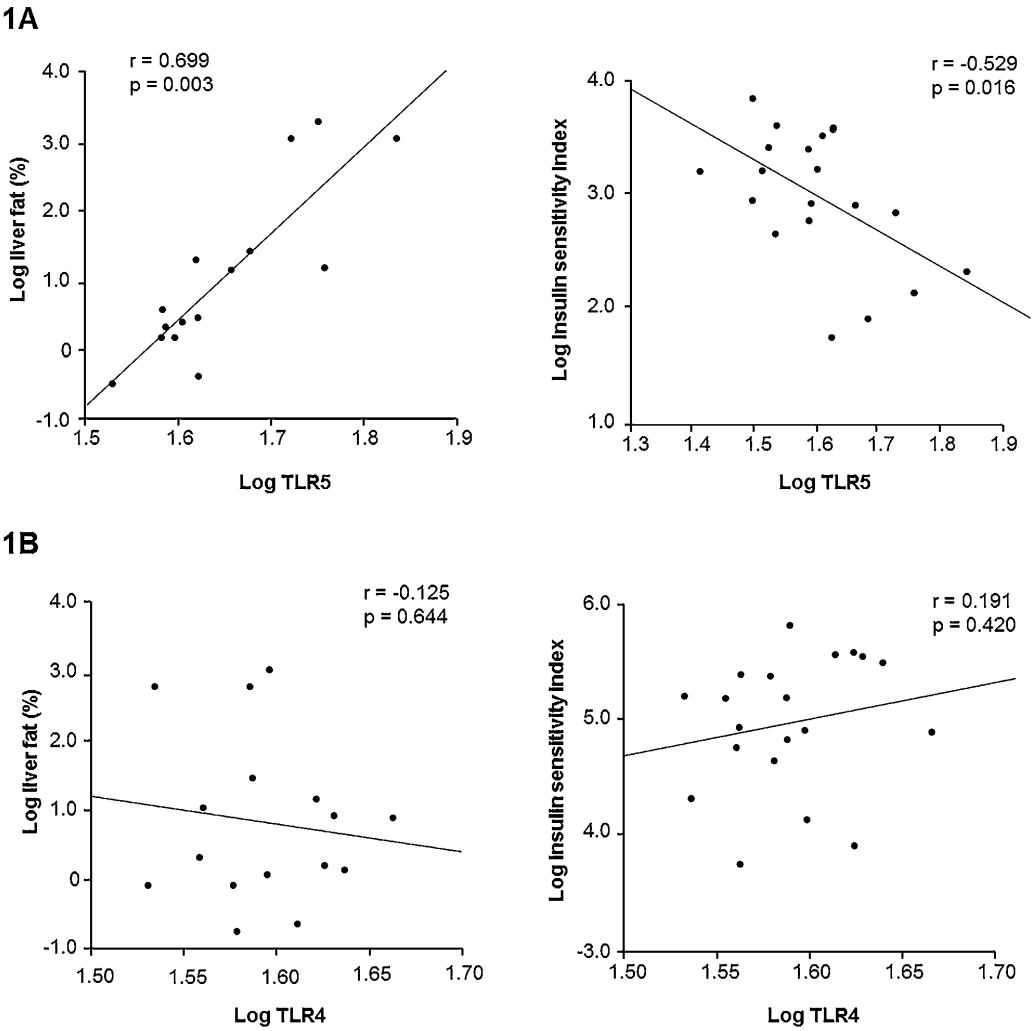

Figure 11. Correlations (n=23) of hepatic fat content and insulin sensitivity with adipose tissue *TLR5* mRNA (A) and *TLR4* mRNA (B). (Munukka *et al.* submitted*)*

4.2.7 The effects of flagellin on hepatocyte fat accumulation and metabolism (IV)

Neither direct FLG nor LPS treatment increased the intracellular lipid abundance in the cultured hepatocytes (Figure 12A). However, the direct exposure of hepatocytes to FLG increased the expression of diglyceride acyltransferase (*DGAT*2) measured as a mRNA fold change after 4 and 24 h ($p < 0.05$). Also LPS increased *DGAT2* mRNA expression after 24 h. DGAT2 participates in the TG synthesis by catalyzing the terminal reaction between diacylglycerol and acyl-CoA and it has been suggested that the inhibition of DGAT2 could serve as a novel target to treat obesity and metabolic disorders (Cases *et al.* 2001). However, it seems an ineffective treatment strategy since the DGAT2 inhibition

actually results in quite mild effects (Chen & Farase 2005). When hepatocytes were exposed to the CCM either from the FLG or LPS-treated adipocytes, *DGAT2* expression increased in hepatocytes at 1 h, but at 4 h only the FLG-treated CCM increased the *DGAT* expression ($p < 0.05$ for all). Only when hepatocytes were exposed for 24 hours to the CCM of FLG-treated adipocytes, intracellular lipid content increased moderately ($p < 0.05$, Figure 12B).

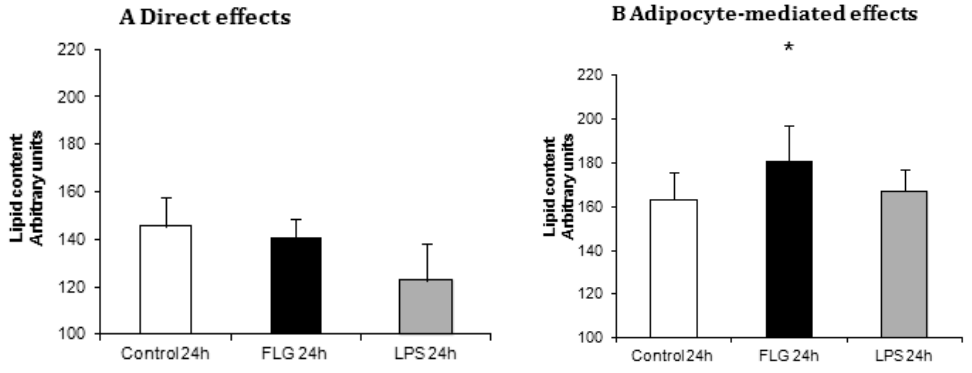

Figure 12. The lipid content of HepG2 cells after A) direct 24 hour exposure to FLG and LPS and B) 24 hour exposure to conditioned culture media derived from FLG and LPS-treated adipocytes. The values are means + SEM (n=12). (Munukka *et al. submitted*)

Glycerol secretion into the culture media increased significantly after the exposure to FLG in adipocytes ($p < 0.05$, Figure 13A). Glycerol degradation and secretion was localized to occur in the lipid droplets (Figure 13B). FLG-treated adipocytes were labeled with TLR5 and perilipin, an antibody against lipid droplet membrane protein, and subjected to confocal imaging. Only a minority of the TLR5 pool was located on cell membranes as the majority localized intracellularly near the nuclei and lipid droplets without any co-localization with those structures. Lipid droplet degradation was observed in 70 % of the FLG-treated adipocytes, and in only 30 % of the untreated control cells after 3 hours (Figure 13B).

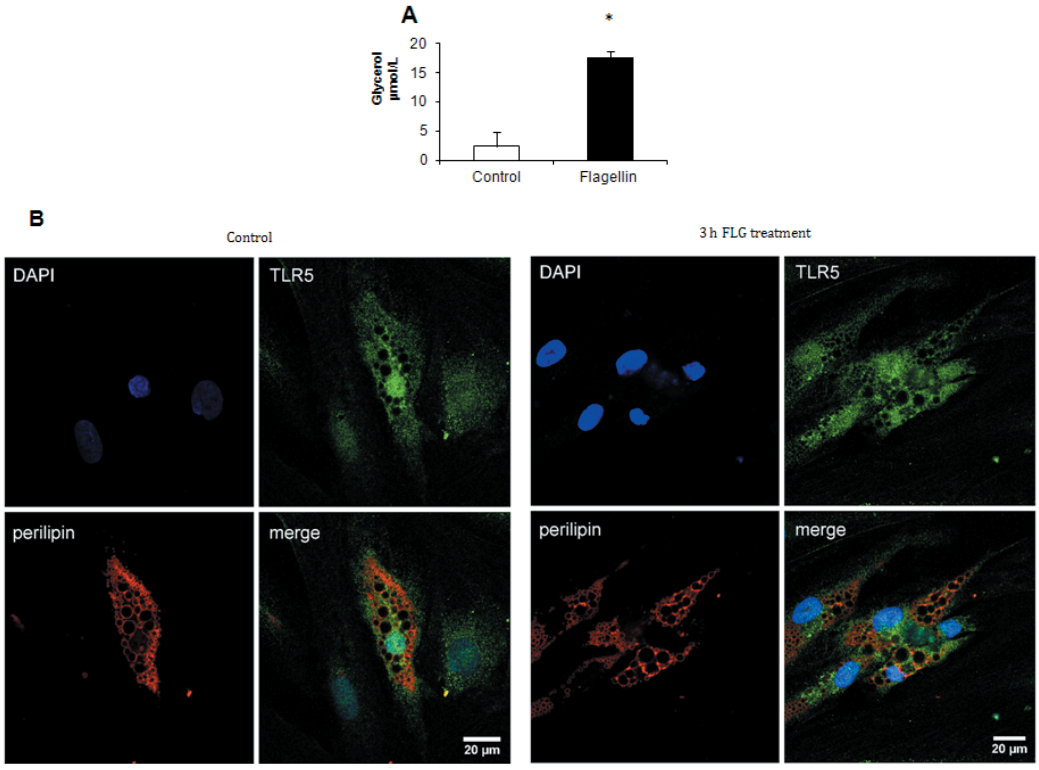

Figure 13. Confocal imaging of glycerol degradation and secretion from the adipocyte lipid droplets in response to FLG treatment. A) Adipocytes treated with FLG and glycerol. The values are means ± SEM (n=4). B) Adipocytes with and without 3 h FLG treatment, labeled with TLR5 and perilipin antibodies. (Munukka *et al. submitted*)

Hence, FLG seems to increase the glycerol secretion from the adipocytes through the increased lipid droplet degradation, which acts as a trigger for the increased hepatic TG synthesis. Hepatic *de novo* fat synthesis pathway enzymes use carbohydrates and glycerol derived from the adipose tissue lipolysis as substrates as shown previously *in vivo* (Franchini *et al.* 2010). Although the direct FLG-exposures seemed to induce the DGAT2 expression, fat accumulation in hepatocytes occurred only when hepatocytes were challenged with the CCM derived from the FLG-treated adipocytes.

4.2.7.1 The effects of FLG and LPS on insulin signaling in hepatocytes (IV)

Moderately decreased Insulin receptor substrate 1 (*IRS1)* expression was observed after the direct exposure of hepatocytes to LPS at 1 h ($p < 0.001$), whereas FLG increased its expression at 4 h ($p = 0.044$). Phosphorylation of Akt downstream in the insulin-signaling pathway increased in response to both LPS and FLG treatments ($p < 0.05$). CCM from the FLG-treated adipocytes slightly decreased *IRS1* mRNA at 24 hours ($p = 0.002$), and phosphorylation of Akt after 30 min and 4 h exposure ($p = 0.017$ and

0.035). The CCM from LPS-treated adipocytes acutely increased phosphoenolpyruvate carboxykinase (*PEPCK)* mRNA, but at 24 hours the media from both LPS and FLG-treated adipocytes decreased it ($p < 0.05$ for all).

Even though the direct FLG and LPS exposures induced *DGAT2* expression, the fat accumulated in the hepatocytes only when they were challenged with the media derived from the FLG-treated adipocytes. This may, at least partly, be due to that while the media derived from FLG-treated adipocytes decreased insulin signaling, the direct exposures to hepatocytes increased it. Previously, the increased Akt phosphorylation in hepatocytes in response to the bacterial surface molecules due to the negative feedback mechanism for TLR-driven inflammation could be detected (Ke *et al.* 2011). The normal insulin signaling together with mitochondrial respiration can retain the correct balance between fat synthesis, oxidation and clearance of TGs, thus preventing the hepatocytes from accumulating fat, when the hepatocytes are directly exposed to the bacterial surface molecules. However, the exact underlying regulatory mechanisms warrant further attention.

4.2.7.2 The effects of FLG and LPS treatments on inflammation and mitochondrial respiratory chain complex subunits in hepatocytes (IV)

Direct exposure of hepatocytes to the FLG increased the cytokine production-related *NF-kB* (p65) expression only at 4 h ($p = 0.002$) as did LPS treatment ($p = 0.006$). *MMP-9* expression could not be reliably detected at 1 h measurement point due to too low detection level. However, LPS increased *MMP-9* mRNA at 4 h ($p = 0.031$) and FLG at 24 h ($p < 0.001$). CCM from both the FLG and LPS-treated adipocytes decreased *NF-κB* (p65) expression slightly at 4 h ($p = 0.024$ and 0.006, respectively), but expression turned to increase in the FLG-media at 24 h ($p = 0.001$). *MMP-9* mRNA levels were increased at 4 h ($p = 0.002$) and then decreased after 24 hours of exposure to FLG ($p = 0.006$). The inflammatory reactive oxygen species (ROS) production increased after exposing the hepatocytes directly to the FLG and LPS ($p <0.05$ for both). In addition, the CCM from LPS-treated adipocytes increased ROS production, and FLG tended to increase it ($p = 0.06$ respectively).

The direct exposure either to FLG or LPS did not exert any major effects on the mitochondrial respiratory chain (MRC) complex subunits except that the LPS treatment increased ATP synthase subunit ATP5A protein expression ($p <0.05$). Contrarily, CCM from the FLG-treated adipocytes decreased ATP5A protein expression, and both FLG and LPS media decreased Cytochrome C oxidase subunit MTCO1 expression ($p <0.05$ for all). Previously, decreased MRC and ATP synthase activity has been reported in NAFLD patients (Perez-Carreras *et al.* 2003). Impaired MRC function is coupled to the increased FA oxidation and constant ROS production during HFA (Begriche *et al.* 2013). Recently, it has been shown that both the TLRs and lipid accumulation by itself increase

the ROS production and inflammation in hepatocytes (Brenner *et al.* 2012). However, results of this study further refine the mechanisms involved as it was found that both the inflammation and glycerol uptake contribute to the fat accumulation in hepatocytes, while the direct exposure of hepatocytes to FLG only increases ROS production maintaining normal intracellular lipid content.

ROS are known for their ability to modify the expression of MMPs (Svineng *et al.* 2008). In this study, FLG and LPS first increased and then decreased the *MMP-9* expression in adipocytes. The result is logical since the extracellular matrix is first degraded and then deposited as a result of proceeding hepatic fibrinogenesis (Svineng *et al.* 2008). On the other hand, the longer direct exposure on the hepatocytes resulted in an increase of *MMP-9* expression indicating that the fibrogenic processes may not be activated. However, recent studies have reported that also TLRs regulate MMP expression and profibrogenic responses and that lipid accumulation itself activates inflammatory responses and profibrogenic gene expression in hepatocytes (Seki & Brenner 2008, Wobser *et al.* 2009).

4.2.8 Limitations of the study

The relatively small number of the participants and the cross-sectional study design limit the conclusions drawn from the human studies. Thus, direct assumptions about the causality and underlying mechanisms cannot be performed. In the future, prospective and intervention studies are needed to confirm the role of the GM in metabolic diseases. In addition, the results of the human studies cannot straightforwardly be generalized to the overall population due to well-known, significant inter-individual variation of the microbiota. However, all study subjects lived in the city of Jyväskylä or its surroundings in Central Finland, i.e., subjects from relatively constricted geographic location was included in study and represent a relatively homogenous population. Hence, the confounding effect of the geographic differences (Yatsunenko *et al.* 2012, Prideaux *et al.* 2013) was minimized, thus strengthening the findings despite of the small study population.

Feces is probably not the most informative sample type in order to evaluate the role of GM in energy metabolism since it is the proximal intestine where both the carbohydrate and fat digestion and uptake occur. Small intestine is also a "hot spot" of the host-microbe interactions as well as the major control point for the glucose and energy homeostasis signaling (Raoult & Henrissat 2014). In addition, it has been criticized that feces do not necessarily represent all the bacterial players habituating the upper parts of GIT (Zoetendal *et al.* 2012). However, still to date most of GM data is received from the analysis of fecal samples due to the inability and impossibility to collect mucosal biopsies from the large population based study cohorts since the ethical codes and compliance hinder the performance of endoscopy for subjects without GI diseases.

In this study, targeted phylogenetic approach (FCM-FISH) was chosen for the assessment of the fecal samples, and detected around 35 % of all the gut bacteria. Hence, it is possible that some important gut bacteria-related findings were neglected due to chosen technique. Though, all the oligonucleotide probes chosen for the study are known to belong to the most common and notable genera and groups in the human GM (Sekirov *et al*. 2010, Rajilic-Stojanovic & de Vos 2014). More comprehensive GMC could have been gained by utilizing phylogenetic microarrays, NGS and metagenomics approaches but they were not available at the moment of microbiota analysis in this study. However, currently it is well known fact that each GMC profiling technique based on 16S rRNA inherently has biases that affect the results (Hugon *et al.* 2013, Brooks *et al.* 2015, http://www.microbiome-standards.org/). Thus, probably the best manner to perform phylogenetic GM profiling would be a combination of multiple techniques in the analysis of same set of samples (Hugon *et al.* 2013).

The *in vitro* -experiments (III and IV) were not performed with the adipocytes separated from the adipose tissue resident in human biopsies. Therefore, the possible contribution of macrophages to the *TLR5* expression levels observed in humans could not be ruled out. However, this setback does not exclude the importance of the relatively moderate but clear effects of the FLG-treated adipocytes on hepatocytes *in vitro* suggesting that the adipocyte-hepatocyte axis constitutes one pathway that leads to the HFA. In addition, the fact that the insulin concentrations were not varied limits the cell culture findings. Therefore, further studies are needed to determine whether the insulin either boosts or hinders the effects of FLG and LPS on hepatocytes.

5. SUMMARY AND CONCLUSIONS

The main findings and conclusions of this thesis work are summarized in Figure 14.

Briefly:

1. Human studies conducted with the Finnish study population strengthened the current scientific consensus on the role of the metabolically aberrant GMC that associates with the metabolic diseases (I-III). As a novel finding, a greater amount of the gram-positive Erec group bacteria belonging to *Firmicutes* phyla associated with the several traits of MetS, higher body fat mass and overall distorted lipid and glucose metabolism.

2. For the first time it was shown that the low fecal abundance of an indicator species of metabolically healthy GM, *F. prausnitzii,* associated with high hepatic fat content (HHFC) in humans. Simultaneously several inflammatory-related genes and cascades related to the leucocyte transendothelial migration and TLR signaling were increased in the adipose tissue of HHFC subjects, suggesting that the adipose tissue inflammation might serve as a possible link between GM and HFA.

3. Aberrant GMC observed in MetS also appeared as higher amounts of gram-negative, γ-*Proteobacteria*, i.e., Enter group in relation to the bacterial groups and species known to be protective such as *F. prausnitzii* (Figure 14). Enter group bacteria harbor the pro-infammatory LPS structure that serves as a ligand for TLR4 that activates the most thoroughly known signaling pathways in innate immunity. Interestingly, many members of the Enter group also harbor FLG, the ligand for TLR5 signaling pathway. Thus, it seems that aberrant GMC may lead to the systemic peripheral inflammation if the state of increased translocation, i.e., dysbiosis prevails in the gut. This further predisposes the host to the abnormal fat accumulation in adipose tissue and/or liver and dysfunctional lipid and glucose metabolism as summarized in Figure 14.

4. *In vitro* experiments in human adipocytes and hepatocytes suggested that the association between GMC and the onset of obesity and metabolic diseases such as NALFD and MetS might involve FLG, FLG-recognizing TLR5 and adipose tissue inflammation. Moreover, the increased abundance of certain flagellated Erec group species such as *E. rectale* may serve as potential activators of TLR5 if dysbiosis prevails in the gut. Finally, the changes in the adipose tissue may further contribute to the obese phenotype with unfavorable metabolism.

5. *In vitro* experiments suggested that the FLG through the increased adipose tissue TLR5 signaling might be involved in the onset and maintenance of obesity and related metabolic changes. The GM-derived components might induce SLGI, which involves at least TLR5 signaling.

6. The findings in humans were corroborated by the *in vitro* results showing that in cultured human adipocytes FLG decreased insulin signaling and increased the inflammatory ROS production and glycerol secretion (Study IV).

In conclusion, the results of this thesis work may suggest that in the future the bacterial-based treatment strategies could serve as novel strategies in order to treat or prevent the metabolic diseases. Specifically, metabolically beneficial bacterial species such as *F. prausnitzii* that in this study associated with the healthy state in humans could be used as a biomarker for a balanced and supportive GMC. The disease-causing mechanisms of the certain members of both Erec and Enter group and their causal role in the onset of metabolic disorders should be further thoroughly studied in order to elucidate the possible preventive or treatment strategies in humans. In addition, the ongoing development of the metagenomic approaches may enable in the future the identification of the specific ”disease-causing” bacterial species and strains within the above-mentioned groups and the assessment of their metabolic capacities. Thus, the rational targeting to their abundance through the evidence-based lifestyle interventions is allowed.

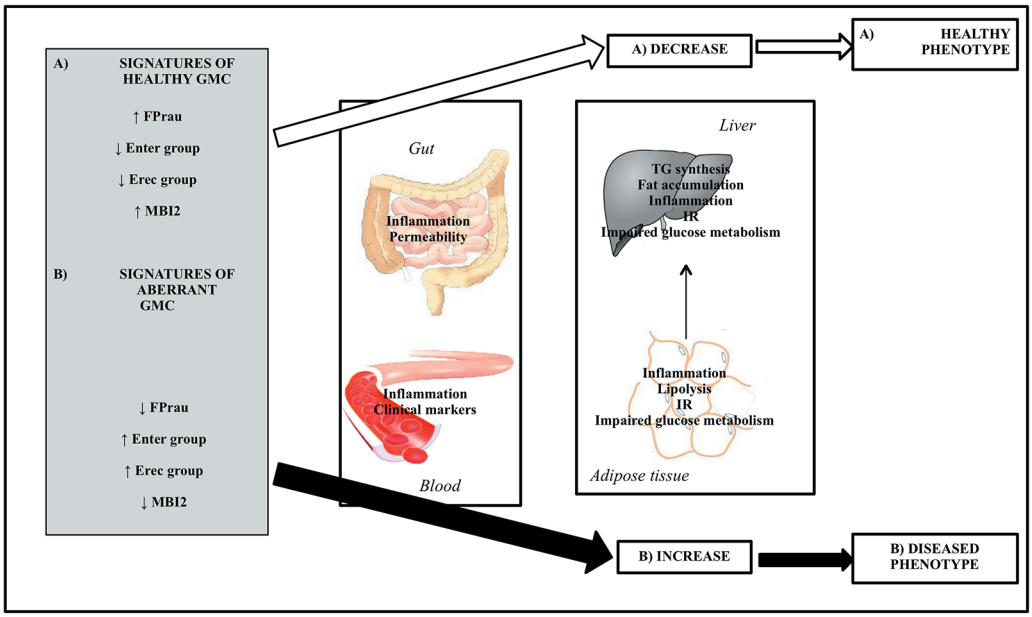

Figure 14. Summary of the results and conclusions of the study. FPrau = *F. prausnitzii*, Enter group = γ-*Proteobacteria*, Erec = *E. rectale – C. coccoides* group, GMC = gut microbiota composition, IR = insulin resistace, MBI2 = Microbial Balance Index2, TG = triglycerides

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