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Effect of an enriched microbial environment on maintenance of gut health and tissue homeostasis in mice.

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Abstract

Introduction: The house mouse, as humans and other animals, have co-evolved with the billions of microbes surrounding them and colonizing their bodies and composing their microbiota. Microbes colonizing the gut, namely the gut microbiota, comprises the largest density of microbes, and the symbiotic relationship between the host and gut microbiota profoundly influence host health. Some anaerobic colonic bacteria have the ability to produce Short-chain fatty acids (SCFAs) through bacterial fermentation. Butyrate is a SCFA shown to pose the main energy source for colonic epithelia, and is important in regulation of gene expression of genes associated with cytokine production, maintenance of tissue homeostasis and immune response in the gut. This knowledge may complicate the interpretation of findings from traditional mice studies using mice housed in laboratory cages under strictly hygienic conditions, deprived of microbial encounters. Thus, to study mice in a more natural, evolutionary habitat poses a novel opportunity to recapitulate realistic responses.

Aim: The overall aim of this project was to study the effects of a microbially rich environment on gut health in mice, by comparing mice housed in a simulated natural habitat or in conventional cages. This project was focused on unravelling possible differences in expression of genes involved in colonic homeostasis and inflammation, and faecal content of SCFAs, in mice housed in the two contrasting environments.

Method: The expression of genes was assessed by quantitative polymerase chain reaction (qPCR) conducted on complementary DNA (cDNA) synthesized from RNA. This RNA was extracted and isolated from the distal part of mice colons. Faecal content of short-chained fatty acids (SCFA) was measured by Gas chromatography (GC), with a flame ionization detector (FID). Various statistical analyses were applied, to determine the differences in the results from the two groups.

Results: The GC-FID analysis showed that butyrate concentration were significantly higher in faeces from lab mice than pen mice. Results from the qPCR showed that *TNFα* and *iNOS* had significant higher expression in distal colon of pen mice, than lab mice. Expression results for the other five genes; *NOD1, NOX1, NOX2* and *MUC2* demonstrated a tendency to be upregulated in colonic mucosa from pen mice compared to lab mice, however not significantly so.

Conclusion: Considering all the results together with the acquired knowledge about the collaborative relation between butyrate and the selected genes, demonstrates that being exposed to an enriched bacterial environment might strengthen and prime the immune system and the mucosal barrier function. Significantly higher expression of *TNFα* and *iNOS* in pen mice colon indicates a prepared immune system with high possibly higher number of primed pro-inflammatory cells such as macrophages. The results may also indicate that this situation improves maintenance of the mucosal barrier function and epithelial integrity. A lower faecal butyrate level in pen mice could be explained by a rapid turnover rate of colonic epithelia, and a corresponding oxidation of butyrate by colonocytes, leading to lower levels of butyrate in faeces and healthy colonocytes.

Sammendrag

Introduksjon: Husmusen, på lik måte som mennesker og andre dyr, har utviklet seg i takt med milliarder av mikrober som omgir dem og som koloniseres i kroppene deres og komponerer mikrobiotaen deres. Mikrober som koloniserer tarmen, kalles tarmens mikrobiota, og dens egenskaper muliggjør et symbiotisk forhold mellom verten og tarmens mikrobiota, noe som påvirker vertens helse i stor grad. Noen anaerobe bakterier i tykktarmen har evnen til å produsere kortkjedede fettsyrer gjennom bakteriell gjæring (fermentering). Butyrat er en slik fettsyre, og er vist å være den viktigste energikilden for tykktarmsepitel, og er viktig i regulering av genuttrykk av gener assosiert med cytokinproduksjon, vedlikehold av vevshomeostase og immunrespons i tarmen. Denne kunnskapen kan komplisere tolkningen av funn fra tradisjonelle musestudier, hvor det blir benyttet mus som er fostret opp i laboratoriebur under strenge hygieniske forhold, uten å bli utsatt for mikrobielt liv. Å studere mus fostret opp i et mer naturlig bakterielt miljø gir en ny mulighet til å gjenskape realistiske resultater innen forskning på sykdomsutvikling og immunsystemet.

Hensikt: Det overordnede målet med dette prosjektet var å studere effektene av et mikrobielt beriket miljø på tarmhelse hos mus, ved å sammenligne mus som er fostret opp i et simulert naturlig habitat (bingemus) eller i delvis sterile konvensjonelle lab-bur (labmus). I dette prosjektet ble det fokusert på å avdekke mulige forskjeller i genuttrykk for gener involvert i vevshomeostase og betennelse i tykktarmen, og fekalt innhold av kortkjedede fettsyrer, hos mus som er fostret opp i de to ulike miljøene.

Metode: Genuttrykk ble studert ved bruk av kvantitativ polymerasekjedereaksjon (qPKR) utført på komplementært DNA (cDNA) syntetisert fra RNA. Dette RNAet ble ekstrahert og isolert fra den distale delen av tykktarm fra mus. Fekalt innhold av kortkjedede fettsyrer ble målt ved gasskromatografi (GK), med en flammeioniseringsdetektor (FID). Ulike statistiske analyser ble utført for å bestemme signifikante forskjeller i resultatene mellom de to gruppene.

Resultater: GK-FID-analysen viste at butyratkonsentrasjonen var betydelig høyere i faeces fra labmus enn bingemus. Resultater fra qPKR viste at *TNFa* og *iNOS* var signifikant høyere uttrykt i distal tykktarm hos bingemus, enn labmus. Genuttrykk for de andre fem genene; *NOD1, NOX1, NOX2* og *MUC2* demonstrerte en tendens til å

bli oppregulert i tykktarm hos bingemus sammenlignet med labmus, men ikke med signifikant forskjell.

Konklusjon: Tatt i betraktning alle resultatene sammen med den ervervede kunnskapen om samarbeidet mellom butyrat og de studerte genene, viser det at å bli utsatt for et beriket bakteriemiljø kan styrke og forberede immunforsvaret og tarmepitelets beskyttende barriere. Signifikant høyere ekspresjon av *TNFα* og *iNOS* i tykktarm hos bingemus kan indikere et godt forberedt immunforsvar med et høyt antall makrofager, i tillegg til opprettholdelse av den beskyttende barrierefunksjonen og integriteten i tarmepitelet. Et lavere fekalt butyratnivå hos bingemus kan forklares ved rask fornyelse av tykktarmsepitel og en tilsvarende nedbryting av butyrat gjort av epitelcellene, fører til lavere nivåer av butyrat i faeces og sunne epitelceller i tykktarmen.

List of abbreviations

DNA - Deoxyribonucleic acid

cDNA - Complementary DNA

gDNA - Genomic DNA

RNA - Ribonucleic acid

mRNA - Messenger RNA

dNTP - Deoxyribonucleotide triphosphate

iNOS - Inducible Nitric Oxide Synthase

NOD1 - Nucleotide-binding oligomerization domain protein 1

NOX1/2 - NADPH oxidase 1 and 2

TNFα - Tumor necrosis factor alpha

MUC2 - Mucin 2

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

LPS - Lipopolysaccharides

PG - Peptidoglycan

NAC - No amplification control

NTC - No template control

PCR - Polymerase chain reaction

qPCR - Quantitative PCR

RNase - Ribonuclease

SCFA - Short chain fatty acid

GC - Gas chromatography

GD-FID - GC flame ionization detector

SD - Standard deviation

SEM - Standard error of the mean

IQR - Interquartile range

CI - Confidence interval

NS - Not significant

HDAC - Histone deacetylase

NF-κB - Nuclear factor kappa light chain enhancer of activated B cells

CD-14 - Cluster of differentiation 14

MAPK - Mitogen-activated protein kinase

GI-system - Gastrointestinal system

NO - Nitric oxide

ROS - Reactive oxygen species

TLR4 - Toll like receptor 4

MD-2 - Lymphocyte antigen 96

OMV - Outer membrane vesicles

NK cells - neutral killer cells

NADPH - Nicotinamide adenine dinucleotide phosphate hydrogen

FAD - Flavin adenine dinucleotide

FMN - Flavin mononucleotide

PRR - Pattern recognizing receptors

NLR - NOD-like receptors

CARD - Caspase activation and recruitment domain

LRR - Leucine rich repeats

RIPK2 - Receptor-interacting serine-threonine kinase 2

TAK1 - Serine/Threonine transforming growth factor b-activated kinase

TNFR -TNF receptor

SODD - Silencer of death domain

TRADD - TNFR-associated death domain

IBD - Inflammatory bowel disease

GPR - G-protein receptor

Cq - Quantitation cycle

LP - Lamina propria

AMP - Antimicrobial peptides

SIgA - Secretory immunoglobulin A

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1.0 Introduction

Our current understanding of immunology and disease development is largely based on studies of inbred strains of the house mice, housed in laboratories under strictly controlled hygiene conditions. However, the common habitat for the house mice is close to normal human and animal activity, where they are exposed to a vast microbial load (Ballenger, 1999). Imagining that scientist may have removed lab mice too far from their natural situation by breeding them in conventional, semi-sterile lab cages. This can shatter the conclusions of animal studies in disease development, as well as translational value to humans, due to lack of interaction between microbes and the immune system as developed through evolution. The consequence of this is the research not being fully reliable. Thus, in the present study, it was recreated a putative natural where the animals are exposed to naturally occurring microbes, attempting to recapitulate the natural symbiosis between microbiota and immunity in the host and simulate more natural responses. Humans are exposed to microbes in their normal environment, and several studies have highlighted that this microbial exposure contribute to the development of the immune system (Huggins et al., 2019). Based on this knowledge, using mice in more natural settings when studying disease development and immune responses, may increase the translation value to humans.

Rebuilt pig pens enriched with material from the farmyard floor was designed at NMBU to resemble a natural habitat for the research mice, where the mice are continuously exposed to environmental microbes. Housing mice in these pens poses an intriguing research opportunity. Such approach could yield research mice with presumably more developed immune system and reveal more realistic physiological responses, in contrast to lab mice bred and raised in semi-sterile conditions.

According to a study by Huggins et al., proposing a hygiene theory, they demonstrated that mice bred in an environment enriched with microbes should have a further developed gut immune system than typical laboratory mice, due to their colonized gut microbiota and beneficial metabolites, contributing to gut health and tissue homeostasis (Huggins et al., 2019). Different hygiene hypotheses have been presented in research over the years, but the most prevalent is that early childhood exposure to infectious agents, such as bacteria, fungi, viruses and parasites, can modulate host immune system development. The gut microbiota comprises the

largest density of microbes and is consequently widely studied. Bacterial colonization of the gut starts at birth and has profound effects on the development and physiology of the host immune system, in addition to health and diseases through life (Bach, 2018). Moreover, several studies have demonstrated that gut microbiota and its metabolites, such as short chain fatty acids (SCFAs), are important contributors in proliferation and differentiation of immune cells (Tanaka & Nakayama, 2017). An important microbial metabolite, butyrate, has the ability to inhibiting gene transcription, and by this regulate some immune responses (Steliou et al., 2012).

By studying gene expression associated with gut health, as well as SCFA content in faecal samples from pen mice and laboratory mice, one could discuss if there are any beneficial connection between gene expression and microbiota metabolites, promoting gut health. A study like this, may emphasize the hygiene theories and the idea of using mice exposed to a microbial environment in studies on intestinal disease and immune system development.

1.1 The Colon

The colon, also known as the large intestine, is a part of the digestive tract. The digestive tract includes the mouth and esophagus, stomach, small-, and large intestine, and rectum. In addition to this, the pancreas, liver and gallbladder are a part of the whole digestive system. The colon is responsible for absorbing water and electrolytes and the removal of waste and nondigestible foods from the body through the rectum (Irving & Catchpole, 1992). In contrast to the small intestine, the colon does not have a major role in absorbing nutrients. Colon is also the site for most of the bacteria that residues in the GI-tract, and they have the ability to digest and ferment foods that are not digested and absorbed in the small intestine. The colon makes up about one-fifth of the length of the digestive tract, reaching from the end of the small intestine (ileocecal valve) and to the rectum (Azzouz & Sharma, 2020). The colon can be divided into five main segments; The cecum, ascending colon, transverse colon, descending colon and the sigmoid colon (Kahai et al., 2020). There are multiple tissue layers making up the colon wall, which is largely common along the GI-tract. Facing towards the lumen is the mucosa, which is made of a single layer of columnar epithelial tissue, dominated by colonocytes and goblet cells. Below the

cell layer, also in mucosa, is the Lamina propria which is rich in immune cells and lymphoid tissue, but also blood vessels that serve the epithelial cells. Below the mucosa is a layer of nerves, blood vessels and connective tissue known as the submucosa. Next layer is known as the muscular layer, with circular and longitudinal muscle cells responsible for contraction of the colon and hence leading to mixing and movement of faecal matter towards the rectum. Serosa is the outermost layer, consisting of a thin layer of squamous epithelial tissue (Ipshita Kak, 2019) (Figure 1).

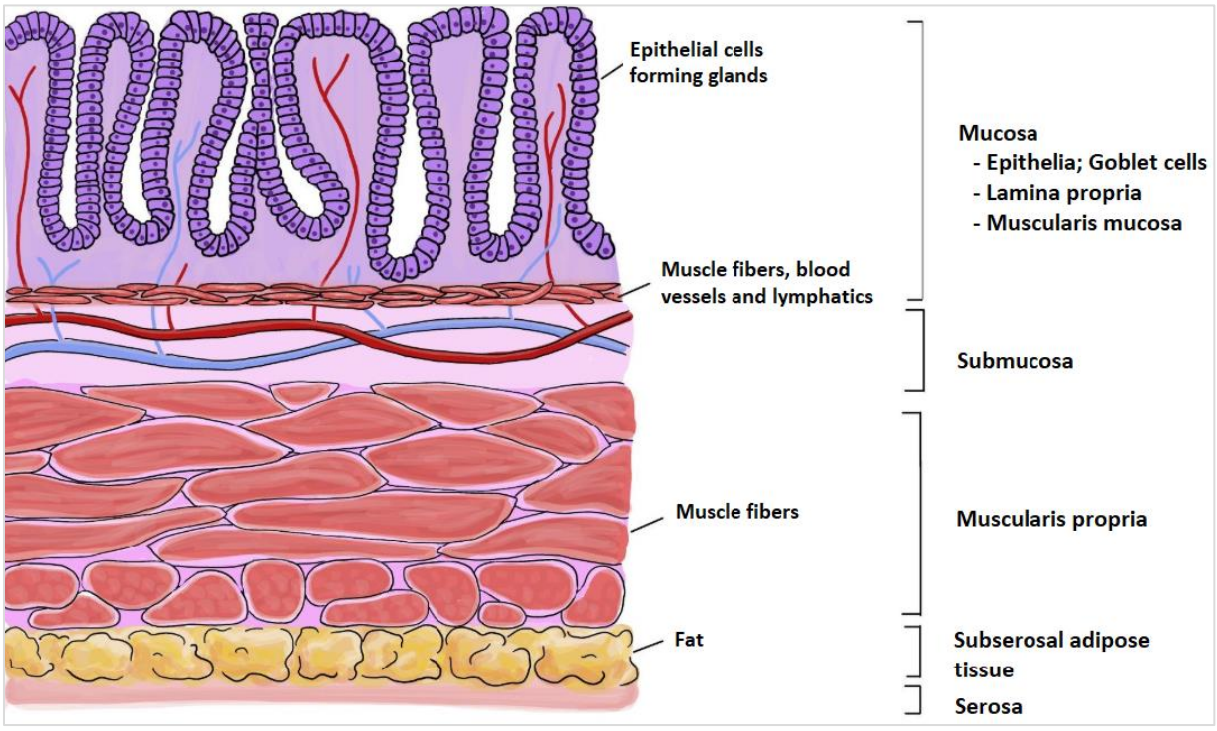

Figure 1 Illustration of the different layers of the colon. Modified figure from Ipshita Kak (Ipshita Kak, 2019).

Mucosa in the small- and the large intestine both contain invaginations. In the small intestine villi stretches toward the lumen to create a very large surface area. In addition, the enterocytes have microvilli to further increase the surface and enhance absorption. In the colon the surface is flatter and have invaginated crypts. These are glandular structures coated with two types of epithelial cells; goblet cells and enterocytes. Goblet cells secretes mucus, consisting of large glycoproteins called mucins (Kathryn A. Knoop & Rodney D. Newberry, 2018). Enterocytes in the colon are also called colonocytes, and are responsible for absorbing water, certain nutrients and electrolytes from passing foods (Miron & Cristea, 2012). Renewal of

colonic epithelia is highly coordinated and regulated and takes place at the bottom of the colonic crypts. Pluripotent stem cells at the crypt base, can be differentiated into several different type of cells. Differentiation means the process in which a cell changes to become tissue specialized. The colonic epithelium has a rapid turnover rate compared to other epithelia in the body. Only 4-5 days after differentiation, there is a controlled apoptosis (cell death) of the cells. The rapid differentiation of stem cells and the corresponding high apoptotic activity of differentiated cells, leads to a high cellular turnover of colonic epithelia (Blachier et al., 2018).

1.1.1 Host defence and immune system of the gut

The defence system of the intestine is usually divided into two parts, including the first and the second line of defense. The first line of defense consists of a physical and a chemical barrier, while the second line of defense includes activation of the immune system when invasive possibly damaging agents crosses the physical barrier.

The colonic mucosa, coated with mucus, serves as a protective barrier between the microbes of the gut lumen and the interior of the host, and represents the physical part of the first line of defense (Kahai et al., 2020). The colon houses a vast load of commensal microorganisms (microbiota), which can be beneficial in mechanisms of metabolism, immunity responses and absorption of nutrients. The mammalian gut can be considered as a complex ecosystem, with gut microbiota existing in homeostasis with the immune system of the host, a symbiotic relationship where both parts benefits (Chow et al., 2010). Homeostasis can be defined as a balanced internal physical and chemical environment, maintaining optimal conditions for tissue specific functions (Lanese, 2009). The mucosal barrier of intestinal epithelia separates the gut microbiota from the host, maintaining the beneficial symbiotic relationship, without harming the host (Okumura & Takeda, 2018).

The mucus layer coating the mucosa is generated by polymerization of mucin proteins, mainly MUC2 in the intestine, secreted by goblet cells. The polymerization transforms the mucins into a gel, composed of oligosaccharides, which provides

chemical protection and constitutes the most important barrier against toxins, antigens and other pathogens in the intestinal lumen. Mucus also has bactericidal abilities, and reduces the amount of direct exposure of bacteria against the epithelial surface (Mello et al., 2012). The epithelial cells of the mucosa have protein-protein networks linking neighbour cells, which fills the intercellular space. These protein networks can form three main types of complexes; adherent junctions, desmosomes and tight junctions (Groschwitz & Hogan, 2009). Colonic epithelial cells are mostly bound to each other with tight junctions, which regulates the paracellular movements across the mucosa barrier (B. Lee et al., 2018).

The mucins forming the intestinal mucus have different properties in the small intestine and the colon. The small intestine only has a one-layered mucus that is not attached to the epithelia and has a structure that allows particles as bacteria to pass through. However, the small intestine mucus has antibacterial content, mostly derived from production of antimicrobial peptides (AMPs) secreted by Paneth cells. The colon has a two-layered mucus, where the inner layer is attached to the epithelia and impermeable, functioning as the protective layer for the epithelial cells. The commensal bacteria in the colon can live in the outer thick mucus layer, which is permeable for bacteria and can provide nutrients for the bacteria residing there. Bacteria thriving in the outer mucus layer, contains glycan-degrading enzymes, and uses them to degrade glycans, releasing monosaccharides as an important energy source (Szabóová et al., 2018).

The chemical barrier of the first line of defence is more prominent in the small intestine than in the colon, due to the small intestine having a high number of Paneth cells, secreting AMPS. Absorptions cells in both the small intestine and the colon can produce some types of AMPs as well, in small amounts. In addition to AMPs, secretory Immunoglobulin A (SIgA) plays an important role of the chemical intestine barrier, especially in the colon. SIgA protects the intestine by keeping pathogenic agents and toxins separated from the intestine epithelia (Ren et al., 2016). In the intestine, there are several indirect mechanisms as well, helping with keeping the gut microbiota under control. Lipocalin-2 (Lcn-2) is a protein with multiple functions, one of them being the ability to inhibit the gut bacteria consumption of iron, controlling their growth. Lcn-2 can be produced both in epithelial cells and immune cells, such as macrophages (Toyonaga et al., 2016).

The second line of defence in the intestine is activated if pathogenic organisms manage to cross the protective mucosal barrier. An inflammatory response is activated by immune cells, in lamina propria (LP). If the intestine wall was damaged in any way, immune cells in the LP, including macrophages, dendritic cells and neutrophil granulocytes which are nonadaptive and phagocytic immune cells, which via pattern recognizing receptors (PRRs) recognize bacterial components on invasive microbes and engulfing them and destroy them in intracellular vesicles. T- and B-lymphocytes are also a part of the immune cells in LP, and are adaptive immune cells (Okumura & Takeda, 2018). B-lymphocytes produce antigens, while T-lymphocytes work as a mediator between the adaptive and the nonadaptive immune system. Signal peptides, called cytokines, are important in the communication between immune cells. Cytokines are often divided into pro- or anti- inflammatory, depending on their ability to increase or decrease the inflammatory response. The nonadaptive and adaptive systems work together to distinguish pathogens and infections in the gut (Sun et al., 2015).

Colonic epithelial homeostasis depends on a strict regulated symbiosis between the commensal microbiota and the immune system of the host. (Robertson et al., 2013). Alterations in the tight junctions of the epithelial barrier, leading to alterations in homeostasis can induce the pathogenesis of several diseases. Studies have shown that any dysfunction in the intestinal epithelial barrier is often associated with inflammatory diseases and other pathological conditions. These dysfunctions can be induced by different factors such as cell stimulation by pathogenic bacterial components like peptidoglycans (PGs) or lipopolysaccharides (LPS), or by proinflammatory cytokine activity. LPS, also called endotoxins, are lipid-soluble outer-membrane components of gram-negative bacteria. PGs are the structural part of the cell walls in both gram-positive and gram-negative bacteria (B. Lee et al., 2018). Sustaining colonic homeostasis depends on maintaining a balance between pro- and anti-inflammatory pathways (Round & Mazmanian, 2009).

1.2 Gut microbiota and its metabolites

Gut microbiota have great importance for the gut health and tissue homeostasis, by stimulating the immune system and adsorption and degrading of nutrients (Azzouz & Sharma, 2020). Mucins contains a large number of clusters of O-linked glycans, which are glycans attached to the hydroxyl oxygen of serine or threonine. Glycans are an important energy source for bacteria. In addition to being able to degrade and extract energy from saccharides transported from the small intestine, the commensal bacteria of the gut have the ability to degrade O-linked glycans of the mucin layer. The energy derived from these glycans can be used by the host to produce more of the protective mucus layer (Roediger, 1980). A vast number of bacteria is housed in the colon, but individual microbiota between hosts varies and permanent changes in the bacteria composition occurs throughout life. An individual's microbiota can vary due to environmental conditions, such as; diets, infections or medications, in addition to change due to age, genetics or hygiene (Dieterich et al., 2018).

Microorganisms housed in the human gut mostly belong to phyla Bacteroidetes, Firmicutes, and Proteobacteria or more rarely to phyla Actinobacteria, Verrumicrobia, Acidobacteria, or Fusobacteria. The colon is dominated by Bacteroidetes and Firmicutes (Dieterich et al., 2018).

Some anaerobic colonic bacteria have the ability to produce various groups of metabolites, by fermenting non-digestible residues of dietary fiber passing though the colon. The bacterial fermentation starts with bacterial enzymes such as polysaccharidases, glycosidases, proteases and peptidases, which breaks down complex polymers such as polysaccharides into sugar components. Sugar components can be fermented by bacteria into SCFAs (Williams et al., 2017). Metabolites such as hydrogen, methane and SCFAs can be fermented, in which SCFAs are the major metabolite group. SCFAs are organic acids, where acetic acid/acetate (C2), propionic acid/propionate (C3) and butyric acid/butyrate (C4) makes up approximately 95% of the SCFA content in the colon (Ríos-Covián et al., 2016). Acetate, propionate and butyrate, have been proved to promote intestinal health by promoting the mucosa barrier function, by regulating permeability of the mucosa barrier (Chambers et al., 2018).

Propionate is one of the SCFAs produced through bacterial fermentation of dietary fibre and is produced mainly by bacteria from *Bacteroidetes* and *Firmicutes phyla*. Propionate has many health benefits, such as the ability to lower blood cholesterol and regulate fat storage, in addition to having anti-cancer and anti-inflammatory properties (den Besten et al., 2013).

Acetate represent the highest percentage of SCFA content in the gut and is mainly produced by bacteria from Bifidobacteriaceae and Lactobacillaceae families. Acetate is an important regulator of intraluminal pH, keeping the gut environment stable, in addition to regulating fat storage and host appetite. When acetate and lactate is produced by e.g. Bifidobacteriaceae and Lactobacillaceae, other gut bacteria in the Firmicutes phyla uses acetate and lactate to produce another SCFA; butyrate (den Besten et al., 2013).

Butyrate production in the colon, is decreased compared to the three other SCFAs. Fermented acetate and lactate can be converted into butyrate, by so-called cross-feeding, which is the mechanism of when one bacterial species lives of the products of other bacterial species (N. W. Smith et al., 2019). Butyrate is produced from two molecules of acetyl-CoA (acetyl group bound to sulphur in a coenzyme A) yielding acetoacetyl-CoA, which is converted into butyryl-CoA. Butyryl-CoA is then yields butyrate through a butyrate kinase (Rivière et al., 2016). There are several types of bacteria that are producers of butyrate in the colon. Some are more researched and well known to be producers than others, such as bacteria from the phylum Firmicutes, typically Faecalibacterium prausnitzii and Clostridium leptum both from the family Ruminococcaceae, and Roseburia spp. Eubacterium rectale from the Lachnospiraceae family also produces butyrate. In addition to these typical microbes, there are many other potential SCFA producers, such as Fusobacteria, Proteobacteria, Bacteroidetes and Actinobacteria (Parada Venegas et al., 2019).

Butyrate is the main energy source of colonocytes (Andoh et al., 1999), and has been studied for its many abilities to promote host intestinal health through multiple tissue specific mechanisms. The most studied mechanisms of butyrate, is its ability to improve colonic epithelial barrier function, by stimulating growth of colonic mucosa and providing energy for colonocytes, which is very important in maintenance of tissue homeostasis (Verbeke et al., 2015). Because of butyrate being the main

energy source for colonocytes, it can promote mucin production by goblet cells, which also improves the barrier function (J. Chen & Vitetta, 2020).

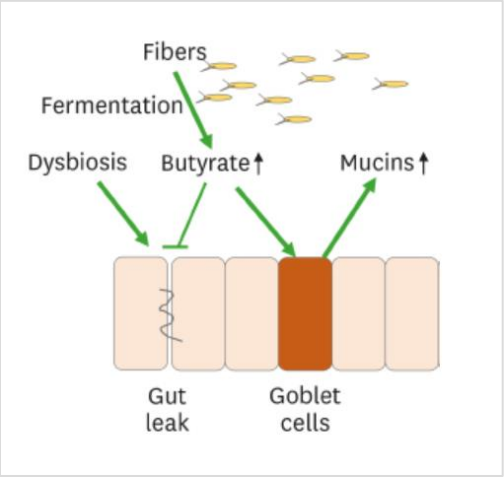

Figure 2 Illustration showing the butyrate-promoted production and secretion of mucins. Modified illustration from J-Chen and Vietta (J. Chen & Vitetta, 2020).

Moreover, butyrate has been shown to inhibit oxidative damage, by affecting expression of genes related to oxidative stress in colon cells (Sauer et al., 2007), and by taking part in inhibiting histone deacetylases (HDAC) (Shao et al., 2004). HDAC are a class of enzymes, that removes acetyl groups from the lysine amino acid on histones, which in turn can lead to a repression of gene expression by the modification of nucleosomes. Inhibiting histone deacetylase by butyrate will therefore do the opposite and stimulate gene expression. Some effects of this is induction of cell cycle arrest, differentiation and/or apoptosis, which may prevent tumour cells from proliferating excessively (Steliou et al., 2012).

1.3 Genes and signaling pathways contributing to intestinal homeostasis

In vivo studies (animal studies) has shown that butyrate can upregulate expression of genes associated with immune response in the colon, in addition to downregulating expression of genes associated with oxidative stress and proliferation of pathogens (Vanhoutvin et al., 2009). Butyrate is also shown to decrease intestinal inflammation by inhibiting the activation of the NF-κB pathway and regulating the production of pro-inflammatory cytokines and anti-inflammatory cytokines (C. Lee et al., 2017). The putative developed microbiota in pen mice is likely to be beneficial for a well-functioning immune system, due to its ability to produce butyrate and stimulate

immune responses. The presence of the microbiota in the gut, is a necessity in development of an well-functioning immune system, that maintain a beneficial symbiosis with the host (Levy et al., 2016).

Based on these studies, among others, it is intriguing to research genes expressed in colonic mucosa and their putative effects on the immune system, both alone and in collaboration with butyrate. In this project, the selected genes include *iNOS, NOD1, NOX1, NOX2, TNFα* and *MUC2.*

1.3.1 Inducible Nitric Oxide Synthase

Inducible nitric oxide synthase (iNOS) is one of three enzymes that produce the reactive molecule nitric oxide (NO). iNOS is encoded by the *iNOS* gene. iNOS is a mediator of unspecific host defence and is often implicated in the process of inflammation and carcinogenesis in the GI-system. *INOS* can be detected in almost all cell types in the GI-system but is dominantly expressed in cells during inflammatory reactions as it is an inflammatory mediator. Studies have shown that *iNOS* is expressed by T cells, macrophages, and mature dendritic cells (Xue et al., 2018). The regulation of *iNOS* expression is complex and includes transcriptional and post-transcriptional mechanisms of modification. The regulation can occur at several sites within the pathways of signal transduction (Shah et al., 2004).

Resting immune cells usually lack iNOS. However, extracellular stimuli can activate signaling pathways that leads to the expression of *iNOS*. Bacteria and fungi can contain cell wall components with the ability to trigger the innate immune system, by inducing a signaling cascade, resulting in the expression of *iNOS*. An example of this; starting with lipopolysaccharide (LPS), which is a cell wall component of gram-negative bacteria. LPS has the ability to bind to special LPS-binding protein, which then delivers LPS on to a high-affinity receptor called CD14 (cluster of differentiation 14). Another receptor; toll like receptor 4 (TLR4) in conjugation with a small protein, lymphocyte antigen 96 (MD-2), interacts with the LPS-CD14 complex and activates a number of signaling pathways. These pathways includes the NF-κB and MAPK-pathways, which leads to activation of transcription of *iNOS* (Taylor & Geller, 2001). LPS has also been suggested to collaborate with the butyrate in controlling immune functions of the host. In cooperative manner, LPS and butyrate mediates the

production of pro-inflammatory cytokines and mediators (Morikawa et al., 2004). The NF-κB pathway consist of the NF-κB protein complex, which is a number of inducible transcription factors, in which mediates gene transcription, cytokine production and cell survival (Lawrence, 2009). The MAPK pathway converts extracellular signals, into cellular responses, such as differentiation and regulation of transcription factors (Cargnello & Roux, 2011).

iNOS contains a N-terminal oxygenase domain, which holds binding sites for arginine. It also contains a C-terminal reductase domain, with the ability to bind NADPH and transfers electrons from NADPH, through FAD and to FMN. When an inflammation occurs, iNOS uses electrons and oxygen from NADPH to oxidize L-arginine into Nitric oxide (NO) and L-citrulline. NO has been shown to be an important signal molecule, having a significant role in host immune response with diverse functions, such as using macrophages to mediate bactericidal and tumoricidal activity (Taylor & Geller, 2001).

NO is a small reactive molecule with has the ability to diffuse through cell walls and interact with cellular compounds. NO is known to have high reactivity and can damage different cell structures, in the case of redox imbalance, which is an imbalance between oxidants and antioxidants in cells. The production of NO was mostly known to inflict damaging processes (Farnese et al., 2016). However, later studies suggest that NO molecules are important signal molecules and has protective effects in the gastrointestinal tract and maintenance of mucosal integrity. Inducible NO production can regulate a number of essential functions in the gastrointestinal tract, especially the large intestinal mucosa. Increased NO production, due to *iNOS* expression is considered a part of the antibacterial immune response in the intestine.(Kolios et al., 2004)

Macrophages, neutrophils and natural killer cells (NK cells), are all cells considered to be a part of the innate immune system. These types of cells use PRRs to detect molecular pattern associated with pathogens. Activated macrophages can inhibit the replication of these recognized pathogens, by secreting effector molecules, such as NO. In addition to function as a toxic defense against infectious pathogens, NO can also regulate growth, death and activity of various immune cells and inflammatory associated cells. These immune and inflammatory associated cells include; macrophages, NK cells, T- lymphocytes and neutrophils (Tripathi et al., 2007).

1.3.2 Nucleotide-binding oligomerization domain protein 1

Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) is a PRR located in the cytoplasm, encoded by the *NOD1* gene. *NOD1* is a member of the NOD-like receptor (NLR) family and play an important role in host innate immune system and inflammatory disease. The ability of NOD1 to recognize bacterial cell wall components, and the signaling pathways activated by the binding of these components (Robertson et al., 2013). The *NOD1* gene is expressed in several different types of cells. *NOD2* is an important ortholog of this gene, and is mostly in monocytes, macrophages and dendritic cells (Kim et al., 2004).

NOD1 is a PRR that can sense conserved motifs in the cell wall of a number of bacteria, such as bacterial peptidoglycans (PGs), and activate intracellular signaling pathways, which induces antimicrobial and proinflammatory responses. NOD-receptors are similar to other PRRs such as TLRs, but unlike TLRs that can sense ligands on the surface of pathogens, NOD1 can recognize invasive products within the bacterial cell wall and cytosol. The NOD1 receptor consists of a NOD-domain (nucleotide-binding oligomerization domain), a CARD-Domain (caspase activation and recruitment domain) and multiple LRRs (leucine rich repeats). NOD2 has two CARD-domains, unlike NOD1 which only consists of one (Kim et al., 2004). The NOD1 receptor is the first line of defense for detection of bacterial PGs within the cytoplasm of epithelial cells. PG binding activates the NFκB signaling pathway leading to transcription of genes, including production of cytokines and chemokines, that contribute to immune responses against recognized pathogenic agents and strengthening the protective epithelial barrier (Robertson et al., 2013).

There are several potential mechanisms that facilitate for bacterial recognition by NOD1. Pathogens can enter the cell due to phagocytosis, bacterial outer membrane vesicles (OMVs) or transmembrane channels or through tight junctions or GAP junctions from a neighbour cell. After entering the cytoplasm, the pathogen can be recognized by a NLR. The LRRs is located at the C-terminal of the receptor, and sense the ligands on the invasive pathogens, while the CARD domain is responsible for protein interaction when initiating the signaling pathway (Caruso et al., 2014).

The NF-κB signaling pathway of NLRs can be quite complex. The activation of NLRs start with the LRR terminal recognizing a pathogen, activating the autoinhibited receptor and leads to self-oligomerization. The activated NLR recruits a receptor-interacting serine/threonine kinase 2 (RIPK2), through CARD-CARD interaction, which is an interaction between the CARD motifs on two proteins, allowing formation of a larges protein complex. Followed by the RIPK2 recruiting a serine/threonine transforming growth factor b-activated kinase (TAK1). The TAK1 is very important in this signaling pathway, because of its ability to activate an IKK kinase complex leading to phosphorylation and ubiquitination of a NF-κB inhibitor. After the ubiquitination of the NF-κB inhibitor, the NF-κB complex is degraded by a proteasome making it possible for the complex to translocate to the nucleus where it can perform downstream transcription of several genes, leading to production of proinflammatory cytokines and antimicrobial peptides (Lappas, 2013). TAK1 can also activate the MAPK pathway (Caruso et al., 2014), which leads to downstream transcription of several genes with the same outcome as in the NF-κB pathway; production of proinflammatory cytokines.

Studies show that not only does NOD1 activate inflammatory immune responses against pathogens by recognizing bacterial PGs, but NOD1 activity might also strengthen the epithelial barrier, by maintaining the integrity of colonic epithelial (G. Y. Chen et al., 2008). NF-κB is shown to be an important regulator of cell survival and maintenance of colon epithelium integrity, especially through activity of the adapter molecule NEMO/IKKγ, a subunit of the IKK complex, which activates NF-κB transcription (Nenci et al., 2007). Epithelium integrity can be described as keeping high quality of the epithelial cell-cell interactions, under perfect conditions, for the epithelial protective barrier to function optimal (Macara et al., 2014).

1.3.3 Tumor Necrosis Factor-alpha

TNFα is another protein coding gene, which encodes a pro-inflammatory cytokine called Tumor necrosis factor alpha (TNFα). The *TNFα* gene is pleiotropic and the encoded cytokine can be produced by different types of cells, and therefore have different roles in cell function, but the TNFα cytokine is produced mainly by macrophages during acute inflammation. TNFα plays an important role in resistance

to infection by regulating a number of cell functions, such as apoptosis, proliferation and differentiation. TNFα is able to promote cell survival by activating NF-κB , and at the same time trigger cell death, by programmed necrosis (Ruder et al., 2019). Macrophages constitutes the first line of defence during inflammation, by being the first to encounter invasive pathogens. Once TNFα is released from macrophages, TNFα activates other immune cells and regulates the production of pro-inflammatory cytokines, thus TNFα is mostly described to function as a pro-inflammatory cytokine (Umare et al., 2014).

TNFα exert its effects on cell functions by binding as a trimer to one of two trans membrane receptors, termed TNFR1 or TNFR2. These receptors belong in the so-called TNF receptor superfamily. TNFR1 is mostly expressed in most types of tissue, whereas TNFR2 is mostly expressed in immune cells, and can be highly regulated. TNF-receptor signal activity has been associated with the pathogenesis of a number of diseases such as diabetes, obesity and Crohn´s disease (Parameswaran & Patial, 2010).

The TNFα trimer binds to the TNFR1 receptor releasing the inhibitory protein Silencer of death domains (SODD), from the intracellular part of the receptor. An adapter protein; TNF receptor-associated death domain (TRADD) binds to the TNFR1, which recruits a couple more proteins, termed RIP-1 and TRAF2 to binds to the receptor. The TRADD-RIP-1-TRAF2 complex is then released from the intracellular domain of the TNFR1, initiating the NF-κB pathway by activating the IKK complex, which leads to phosphorylation of an inhibitor of nuclear factor kappa (IκBα) protein. These mechanisms facilitates for the NF-κB activation and trans location, inducing gene transcription, which leads to proliferation of macrophages and inflammatory response to retain tissue homeostasis (Parameswaran & Patial, 2010). TNFα can be described as a immunomodulator, and induces both systemic and local acute responses by secretion of pro-inflammatory cytokines. In patients with inflammatory bowel disease (IBD), TNFα cytokine levels in stool are often increased, confirming the immune response in inflammatory conditions in the intestine (Andoh et al., 1999).

1.3.4 NADPH oxidase 1 and 2

NOX1 and *NOX2* are homologues and protein coding genes, encoding membrane bound enzymes termed NADPH oxidase isoform 1 and NADPH oxidase isoform 2, also referred to as NOX1 and NOX2 (Darby & Jones, 2015). These proteins mediate the transportation of electrons from NADPH to molecular oxygen through biological membranes, reducing oxygen to generate hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals (OH), all referred to as reactive oxygen species (ROS). Excessive produced ROS is known to be associated with oxidative stress and pathology, such as DNA, lipid and protein damaging. Studies has shown that an overproduction of ROS (oxidative stress) may cause tissue damage, through mutations, that might lead to cancer development (Rosignoli et al., 2001). However, ROS generation by NOX1 is recently shown to be important messengers in cellular signaling, inducing processes such as differentiation and proliferation of normal stem cells (Karen Bedard, and Karl-Heinz Krause, 2007). Isoforms of NOX have multiple functions that are important for normal physiologic functions in the gut (Pizzolla et al., 2012). NOX1 is mainly expressed in epithelia and generate more ROS than NOX2, which is mainly expressed in phagocytic cells. Studies show that bacterial products cooperating with pro-inflammatory cytokines, such as TNFα, has the ability to stimulate the NOX-expression and NOX induced ROS production in colon epithelial cells (Ramonaite et al., 2014).

In the gut, NOX1 is the main NAPDH oxidase isoform and is regulated by subunits of NOXs, such as NOXO1. It is assumed that NOX1 activity, regulated by NOXO1 in the gut mediates epithelial homeostasis and cell renewal (Moll et al., 2018). Recent studies show that *NOX1* is highly expressed in colon epithelia, and ROS produced by NOX1 contribute to production of pro-inflammatory cytokines and may be necessary for normal intestinal cell proliferation. It is also implicated that the NOX1 enzyme is crucial in injury response and maintenance of colon tissue health (Darby & Jones, 2015).

NOX2 is often described as the phagocyte NADPH oxidase, because this isoform was first known in neutrophils and macrophages. NOX2 expression has been shown in some studies, to be distributed in total mRNA of various types of tissue, including the small intestine and colon (Karen Bedard, and Karl-Heinz Krause, 2007). When

NOX2 is activated by microbes or cytokines, it produces ROS. If this NOX2 induced ROS production is not regulated, it may lead to tissue damage (Pizzolla et al., 2012). NOX2 beneficial activity in the intestine is not well known, but recent studies has shown that NOX2 activity is crucial in bacterial killing in neutrophils, especially in patients with illnesses causing high susceptibility to infections, such as Chronic Granulomatous Disease (Brown & Griendling, 2009). Also NOX2 has the ability to regulate NF-κB activation in macrophages, and limit inflammation in response to tissue injury (Singel & Segal, 2016)

1.3.5 Mucin2

Mucin2, Oligomeric gel forming, also known as MUC2 is a protein encoded by the *MUC2* gene. Mucins are glycoproteins produced by many different types of epithelial cells. The Mucin2 protein is secreted by goblet cells in the colon and makes up both the outer and inner mucus layer of the colon. Colonic mucus consist of several types of mucins, with MUC2 as the most prominent in both healthy and inflamed colon (Roediger, 1980).

MUC2 proteins creates the mucus barrier, that protects the gut lumen. The centre domain of the Mucin protein consists of repeats of threonine, serine and proline, whereas the number of copies differ in each individual. This composition gives the protein a high capacity of binding water, helping with the forming of the gel. The gel barrier made by the MUC2 protein coats intestinal epithelial, protecting against invasive bacteria and infections agents (Johansson et al., 2011). Mice deficient of *MUC2* expression has been proved to spontaneously develop colitis (colonic inflammation) and colon cancer, suggesting that the colonic mucosal layer is important in maintenance of colonic homeostasis and protection from external damage (Van der Sluis et al., 2006).

The commensal bacteria in the gut uses its energy from glycans in the mucus to produce short chained fatty acids (SCFAs) by fermenting. These SCFAs can diffuse through the mucus layer and be used as an energy source for colonocytes, by oxidation (Roediger, 1980). A major part of the energy supply for the colon epithelial cells come from SCFAs, mostly acetate and butyrate. G-protein receptors in colonic

epithelia, such as GPR41 and GPR43, allows SCFAs to bind to colonocytes, which activates the cells. This connection supports the assumed effect that SCFAs, such as butyrate, has on the differentiation, health and growth of intestine epithelial cells (Johansson et al., 2011).

1.4 Aims of the present project

The aim of this project was to study the effects of an enriched bacterial environment on maintenance of gut health and tissue homeostasis in mice, by looking at the connection between gene expression in colonic mucosa and faecal butyrate content. Six genes were selected, based on earlier studies presenting their role in maintaining colon homeostasis. These six genes included; *iNOS, NOD1, NOX1, NOX2, TNFα* and *MUC2*.

To research gene expression and faecal butyrate content, colons and faeces samples were collected from house mice (mus musculus). The house mice were divided in two groups, including mice bred and raised in typical semi-sterile laboratory cages, referred to as lab mice, and mice bred and raised in designed pens, pen mice. The pens were designed to resemble the common habitat for the house mouse, with a woodchip bedding, enriched with organic soil and faecal content from various farm animals.

Specific research questions to be assessed in the present project;

Does housing of mice in an diverse and enriched microbially environment have an effect on faecal SCFA concentration? Does housing of mice in an diverse and enriched microbially environment have an impact on relative expression of genes associated with gut homeostasis and health? Does housing of mice in an diverse and enriched microbially environment influence the putative beneficial cooperation between genes associated with gut homeostasis and butyrate?

2.0 Methods

In vitro techniques were applied to study the effects of an enriched bacterial environment on colon health. The techniques described in the current section were conducted on colon tissue and stool samples from mice housed in pens and semi-sterile lab cages.

2.1 Gene expression

To study the effects of an microbially diverse environment on gut health, gene expression analysis of genes involved in inflammatory pathways and maintenance of gut health was conducted. The expression of genes was assessed by quantitative polymerase chain reaction (qPCR) conducted on complementary DNA (cDNA) synthesized from RNA. This RNA was extracted and isolated from the distal part of mice colons.

2.1.1 Preparation of mucosa samples

The starting material for the gene expression analyses was full-length colons harvested from 20 male mice, 10 mice from pens filled with barn material, and 10 from semi-sterile lab cages, referred to respectively as pen and lab mice. The colons were submerged in RNAlater (Sigma-Aldrich) for 24 hours before they were stored at -80 °C until further handling.

The mucosa tissue from the colons were sampled using a scraping technique. Briefly, the colons were cut open longitudinally. The luminal side of a colon was scraped with a glass microscope slide, and mucosa from the distal 1/3rd part of the colon was collected in Eppendorf tubes with RNAlater. The mucosa samples were then stored at -20 °C until RNA isolation.

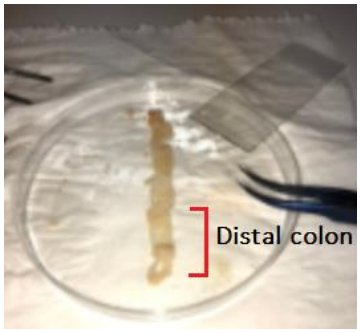

Figure 3 Picture from the present project. Showing a mice colon, stretched out and cut open longitudinally, pointing out the distal part of the colon.

2.1.2 RNA isolation

Total RNA was isolated from the mucosa samples using Nucleospin RNA/Protein Purification kit, according to manufacturer´s manual (Macherey-Nagel, 2019).

To isolate total RNA from colon mucosa tissue, a technique using silica spin column technology was conducted. Using filter columns that binds nucleic acids under specific conditions, resulting in isolated total RNA.

Pieces of mucosa tissue was weighed, and between 15-30 mg was transferred to a mix of lysis buffer and β-mercaptoethanol that disrupts cell membranes and enables access to RNA. The mix of tissue sample and lysis buffers was homogenized using syringes and needles. The homogenized lysate was transferred to NucleoSpin filter-tubes containing silica membranes, and was then filtrated by centrifuging. The supernatant was collected and used further on in the purification. The filtrated samples were then mixed with 70% ethanol to adjust pH to optimize RNA binding conditions, followed by a transfer of the samples to new NucleoSpin columns and centrifugation to bind RNA to the silica membranes of the columns. Using a desalt-buffer and centrifugation, the RNA molecules was washed, removing smaller molecules and salts. A DNA-digesting enzyme; rDNase and a reaction buffer was added to the samples and incubated in room temperature to remove contaminating genomic DNA (gDNA). This is important because the qPCR reaction cannot distinguish between cDNA synthesized by RT-PCR and contaminating gDNA. Contamination of gDNA can lead to overestimation (false positives) of the amount of RNA present in a sample (Añez-Lingerfelt et al., 2009). Using washing buffer and centrifugation in multiple steps, the column-membranes containing RNA were washed and dried. Last step of the RNA isolation was eluting the pure isolated RNA, adding only RNase-free water to the columns and centrifuging. Total RNA was stored at -80°C.

2.1.3 RNA quality and quantification

It is common in molecular biology to perform quantification of nucleic acids in various samples, to determine concentration and purity of Protein, RNA or DNA present. This is especially important before conduction a PCR. It is important because without knowing the amount and quality of the RNA used in the PCR reaction, the gene expression data won't be reliable (Udvardi et al., 2008). NanoDrop and Bioanalyzer is examples of instruments designed for this purpose. In this project, RNA concentration and quality was assessed using Nanodrop and Bioanayzer.

NanoDrop 2000c is a full-spectrum UV spectrophotometer, which in collaboration with the *NanoDrop 2000™* software, can provide concentration values of RNA in ng/µl from a small volume of sample (0.5-2.0µl). In addition to RNA concentration, a measurement of purity is provided, so-called 260/230 and 280/260 ratios. NanoDrop 2000c determine RNA concentration and quality by measuring absorbance at wavelengths of 230, 260 and 280nm. 260nm represents the measurements of RNA, meaning that the 260/230 and 280/260 values provides information about the relation between contaminants and RNA. Deviant 260/230 ratios could mean contamination of salt compounds, such as residues from the lysis buffer used in RNA isolation, whereas abnormal 280/260 ratios usually indicate contamination of proteins or other reagents such as phenol. 260/230 ratios of 2.0 – 2.2 and around 2.1 for 280/260 is generally considered indicative of acceptable RNA quality. Deviant results can also occur due to measurement issues (Matlock, 2015).

Bioanalyzer is an instrument that can provide quality measurements for DNA, Protein and RNA in various samples, including indications on level of degradation. Knowing that the RNA is intact and not degraded before performing expression analyses, is crucial to get reliable expression data. The Bioanalyzer is a chip-based electrophoresis system, that includes the main instrument, data software, a specific chip and reagents for DNA, RNA or protein analysis. The principle of the chip-based analysis is the same as a conventional gel-electrophoresis, which is a method used to separate fragments of macromolecules based on size and positive/negative charge. Bioanalyzer is more efficient and with a lower risk of errors than conventional gel-electrophoresis, due to it being less hands-on with samples and gel-preparation.

As in gel-electrophoresis, Nucleic acid-concentrations is determined by detecting fluorescence molecules bound to RNA or DNA strands. The detected fluorescens is translated into bands in gel-like images and peaks in electropherograms. The Bioanalyzer detects fluorescens from certain structural ribosomal RNA (rRNA), 18S rRNA and 28S rRNA ratios, and illustrates them as peaks in an electropherograms, one for each RNA sample (Odilo Mueller, 2016), from this one can draw assumptions on RNA quality.

Bioanalyzer analysis was conducted using the manufacturer´s manual. Reagents stored in -20 °C was thawed and kept on ice when preparing the reaction mixes. Reagents used in the analysis include a ladder (standard), which is used as a reference for the gel bands, dH2O, electrophoresis gel, and a gel/dye mix prepared beforehand, containing fluorescens molecules.

A chip (figure 4), specific for RNA reagents was used and first loaded with dH_2O to blank the instrument before measuring the RNA samples. After the dH_2O blanking, the chip was then emptied and placed in the priming station to load the gel/dye mix, and then reaction reagents and RNA samples.

Before loading the RNA samples on to the chip, all wells of the chip was filled with a fluent electrophoresis-gel in a specific marked well (black G). By using a customized chip priming station (Agilent), It is possible to load and spread an electrophoresis-gel in all the wells of the chip, using an attached syringe. Further on, the gel/dye-mix and the ladder was applied onto the chip in their respective marked wells (see figure 3). Followed by applying one RNA sample in each of the 12 remaining numbered wells of the chip.

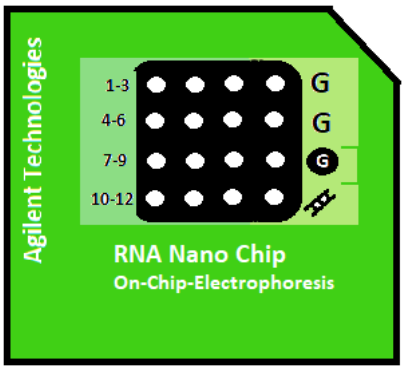

Figure 4 Illustration of the Agilent RNA 6000 Nano Chip, used in the analysis.

Bioanalyzer data consists of bonds on a gel-like image for each sample, and peaks in electropherograms based on these. The peaks in the electropherograms represents detection of 18S- and 28S ribosomal RNA (rRNA).18s and 28s rRNA are structural rRNAs, and detection of both these peaks indicates that the total RNA is intact and not degraded. The ideal electropherogram has both peaks detected, with the 28S peak-area a bit bigger than the 18S. This because 28S rRNA degrades easier than 18S, and a large 28S peak-area therefore indicates intact RNA. The figures below presents the electropherograms collected from the bioanalyzer analysis of the total RNA samples.

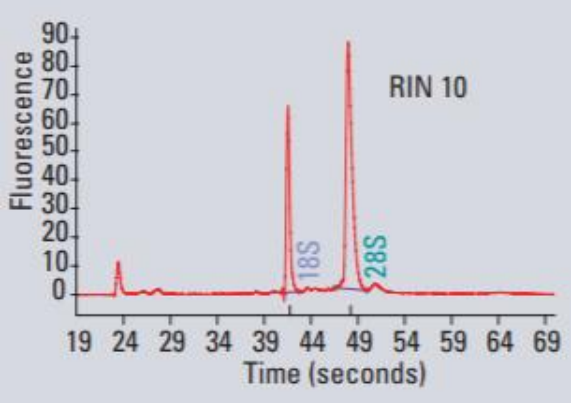

Figure 5 Illustration of an ideal electropherogram of intact RNA, detected by a Bioanalyzer system. X-axis showing time, and y-axis showing fluorescens. (Odilo Mueller, 2016)

After analysing quality and concentration of total RNA using NanoDrop and Bioanalyzer, the total RNA was synthesized to cDNA to be used in qPCR, looking at gene expression.

2.1.4 cDNA synthesis

RNA molecules are very unstable and can easily be degraded by surrounding enzymes; RNases. DNA are much more stable than RNA, in is not as easy degraded, therefore it is common to synthesize cDNA from RNA using reverse transcriptase (RT) and PCR, referred to as RT-PCR. RT is an enzyme, a RNA-dependent DNA polymerase, which acts on single stranded mRNA and uses a RNA template and a primer that is complementary to the 3´ end of the RNA to synthesize cDNA (Rio, 2014). It is easier to research the information of the RNA without the high risk of degrading, by using the cDNA further on in qPCR gene expression analysis.

cDNA synthesis from RNA extracts was conducted using iScript cDNA Synthesis Kit (Bio-Rad). All RNA samples were standardized by making aliquots of 100 or 200ng/µl, depending on the RNA concentration of each sample, measured using NanoDrop 2000.

Depending on the number of samples; the needed amount of each reagent was calculated. Due to possible errors while pipetting, one extra reaction was taken into account, in addition to reaction with a no amplification control (NAC), which is a control sample containing all qPCR reagents, except from transcriptase enzyme. A reaction without cDNA template (No template control; NTC) was also included. All reagents were provided in the assay kit. For each reaction the following was combined;

Table 1 Mixing ration of reactants added for each reaction of the qPCR run.

Volume	Reagent
4 µl	5x iScript reaction mix
1 µl	iScript reverse transcriptase (enzyme)
11 or 7 µl (Depending on the RNA template volume)	Nuclease-free water
4-8 µl (0,8 µg total RNA)	RNA template
20 µl	Total volume

First, the Nuclease-free water and reaction mix for all samples, including NAC and NTC, were combined in an Eppendorf tube.

The NAC was prepared by adding 15 µl of the combined water and reaction mix to an empty PCR tube, followed by adding 1µl of nuclease-free water. The NAC was prepared without enzyme, and had a mix of 1µl RNA from four randomly selected samples as template.

Using the original Eppendorf tube containing water and reaction mix, 1µl of enzyme per sample was added to the tube, and mixed by pipetting. Depending on the starting aliquot concentration of each samples, 16µl or 12µl of the solution was distributed into empty PCR tubes. Followed by adding 4 or 8µl of RNA template to each tube.

The complete mix was then incubated in a PCR instrument, and the synthesized cDNA was diluted 1:5, in DEPC-treated (Diethyl pyrocarbonate-treated) water, and then stored at -20 °C until qPCR analysis.

Table 2 Showing the temperature cycles used in the qPCR runs of this experiment.

Step	Temperature	Duration
Primer annealing	25°C	5 min
cDNA synthesis	42°C	30 min
cDNA synthesis termination	85°C	5 min
Hold	4°C	+∞

2.1.5 Quantitative PCR

To research the putative beneficial effect of exposure to a bacterial environment on promoting gut health, a qPCR analysis was conducted to look at the expression of genes associated with gut health and immune response, in the colon mucosa.

Quantitative PCR (qPCR) is a laboratory technique used to monitor and detect amplifications of a targeted region of a DNA molecule (amplicon) during a polymerase chain reaction (PCR) reaction. The qPCR analysis differ from the conventional PCR, by detecting amplifications in real time, while conventional PCR only calculates the total amplifications at the end of the reaction (Michael W. Pfaffl, 2004).

The principle of qPCR is as following; First, a reaction mix is combined including DNA polymerase (enzyme), dNTPs (deoxyribonucleotide triphosphate), specific primers to fit the target cDNA sequence, fluorescens-generating dye and cDNA template. During the qPCR reaction, with a given temperature program repeated in cycles, the cDNA will be amplified by the DNA polymerase. The specific primers contributes to the amplification by binding to single-stranded DNA initiating the amplification activity of the polymerase. The fluorescens-dye generates some fluorescence by itself, but the signal increases significantly when binding to double-stranded DNA. The amount of cDNA will increase during each cycle, together with the fluorescens signal. When the increase in fluorescens signal per cycle reaches an exponential phase, that is when the signal first is detected as an actual cDNA amplification, and not just background noise (baseline signal). The qPCR data provides Cq-values (cycle quantification), which is represents the number of cycles necessary to reach the exponential phase in the reaction, also called the cycle threshold (Ct) (Arya et al., 2005).

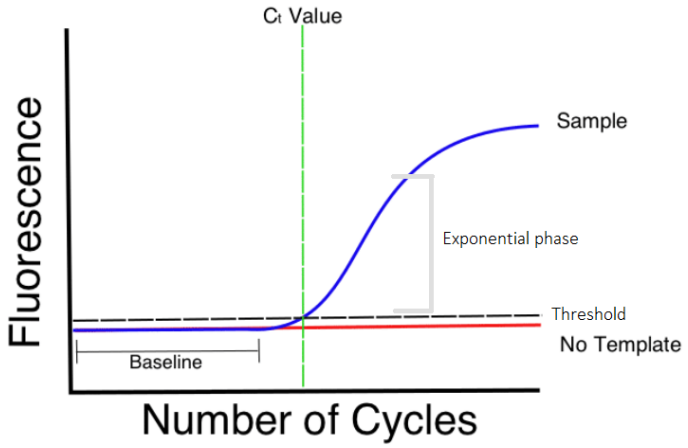

Figure 6 The increase in detected fluorescence signal during the qPCR reaction. Pointing out the exponential phase of the curve, with the Ct value detected at the quantification threshold (Dr Nick Oswald, 2015)

In this project, 5x HOT FIREPol EvaGreen qPCR Supermix with the accompanying protocol (Solis Biodyne) was used for the qPCR reaction. EvaGreen supermix is a ready-to-use reaction solution, containing EvaGreen-fluorescence dye in addition to combining all the other components necessary to perform qPCR, including; DNA polymerase and dNTPs. For the qPCR reaction, the cDNA samples were mixed with H_2O and the primers for the gene of interest, in addition to the EvaGreen qPCR supermix.

The plate setup for the qPCR runs included three technical replicates for each biological sample. NTC and NAC were included to assess possible gDNA contamination and primer dimer formation. NTC and NAC samples were run in duplicates.

To determine the plate setup and confirm the qPCR cycle setup, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was included as the house keeping gene (reference gene). *GAPDH* is a gene associated with carbohydrate metabolism, and is usually expressed in all cells (Robert D Barber et al., 2005). The following genes were selected due to their assumed importance in the promotion of intestinal health and/or association with butyrate-induced mechanisms in the colon; *INOS, NOX1, NOX2, TNFα, Muc2* and *NOD1*. Primers used in this project had been designed using a primer-BLAST tool (NCBI, primer-BLAST tool), and tested previously.

Working in a nuclease-free area, EvaGreen supermix and cDNA template samples was kept on ice as the 96 well qPCR-plate was prepared. A sterile Eppendorf tube was prepared with the total amount of EvaGreen supermix, H_2O and gene-specific forward- and reverse primer, for all samples. Technical triplicates of 20 cDNA samples (ID #41-60) is a total of 60 samples. The 60 cDNA samples, in addition to four controls (two NACs and two NTCs) and two extra reaction in case of pipetting inaccuracy, added up to 66 reactions to prepare in the tube. The qPCR plate was prepared, distributing 7µl of the reagent-mix from the Eppendorf tube in each necessary well on the qPCR plate. Depending on the plate setup, 3µl of each cDNA sample was added to the plate as triplicates, making the total volume in each well 10µl. In four of the wells, 3µl of water for the NTC and 3µl of NAC was added in two wells each, instead of cDNA template.

Table 3 Volume of each qPCR reagent per reaction.

Component	Volume (µl)
5x HOT FIREPol® EvaGreen® qPCR Supermix	2,0
Primer Forward	0,2
Primer Reverse	0,2
cDNA template	3,0
H2O	4,6
Total	**10**

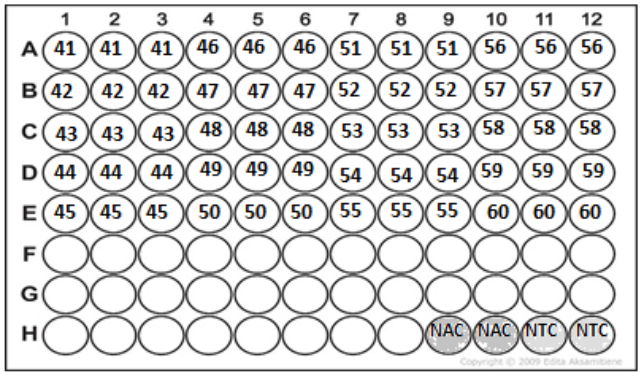

Figure 7 The plate design for the qPCR run. Showing were the technical triplets of each sample were placed on the PCR plate, in addition to the No amplicon control (NAC) and the No template control (NTC).

Each gene of interest analysed in the qPCR, have a specific primer added in the reaction mix. Each of these primers has different annealing temperatures (Tm), depending on the length and composition of the primers. Other than the annealing temperature, all other settings for the qPCR run is similar for all primers.

Table 4 Showing each reaction cycle of the qPCR reaction. [1]Activation of the polymerase enzyme. [2]promote primer binding to the template. [3] the synthesis of DNA by DNA polymerase.

Cycle step	Temperature	Time		Number of cycles
Initial activation[1]	95°C	12 min		1
Denaturation	95°C	15 sec		40
Annealing[2]	60-65°C (Varying Tm)	20 – 30 sec		
Elongation[3]	72°C	20-30 sec		

2.1.6 Processing of qPCR data

The qPCR run gave a large dataset of Cq values (measured fluorescens per cycle) for each technical replicate. The Roche LightCycler qPCR provide raw data in txt-files, that was manually exported into excel files. To analyse these Cq values, a LinRegPCR January 2020 software was used, to calculate the amplification efficiency of each sample and to determine baselines. The efficiency is calculated from slope of the amplification curve in the exponential phase.

Data from the qPCR analysis is calculated as relative quantification, that means that the values given by the qPCR and LinRegPCR is the relative calculated gene expression of a target gene, no matter what the cDNA amount in the original samples were. This is why it is important to normalize the raw data from the analysis, so that the expression levels presented in a study is reliable according to cDNA amount. The qPCR analyse also provides efficiency values for each sample, which is defined as the expression ratio, or the increase in amplicons per cycle (Svec et al., 2015). Normalization using a House Keeping (HK) gene helps to control/even out the amount of molecules in the starting material of each sample and amplification efficiency (Goni et al., 2009).

The normalization was conducted on the average Cq-value of three technical replicates from each individual. The *Pfaffl-method* (Michael W. Pfaffl, 2004) was applied as the normalization, which is a calculation of gene expression data while accounting for differences in primer efficiencies. The Cq values of the equation are form the same sample, from one individual, but from different qPCR runs/reactions with different specific primers for HK- and target gene;

$$N = \frac{(E_{\text{target gene}})^{(-Cq\ \text{target gene})}}{(E_{\text{HK gene}})^{(-Cq\ \text{HK gene})}}$$

N: normalized mRNA-expression of the target gene for each individual sample. E: efficiency/expression ratio of both HK gene and target gene. Cq: the cycle number of which fluorescens can be detected (quantitation cycle).

2.2 SCFA analysis

Faecal content of short-chained fatty acids (SCFA) was measured by Gas chromatography (GC), with a flame ionization detector (FID).

GC is a technique used to separate volatile compounds, which is compounds that are easily vaporized in room temperature. The technique uses a carrier gas as a so-called mobile phase, and a liquid stationary phase to separate the volatile compounds of a sample. The sample to be separated is injected in one end of the column, where the mobile phase, in this case helium, mixes with the sample to transport it along the column. The column, which is coated with a high-boiling liquid, in this case a *Stabile-wax DA-column* serves as a stationary phase. This is a stationary phase that has been specially deactivated to analyse acidic compounds (Banel & Zygmunt, 2011), serves as the stationary phase. As the carrier gas and the sample moves along the column, the compounds of the sample that has strong attraction and form strong bonds to the liquid coating, will move slower through the stationary phase, than those who form weaker bonds. A detector, often a flame ionization detector (FID), located at the end of the column detects the type of compound and the quantity, by detecting the retention time of each compound. The time each compound uses to pass from the injection point to the detector is referred to as retention time. The FID consists of a H_2/air flame that breaks down the organic molecules of the sample passing through, producing ions which is detected by a collector plate.

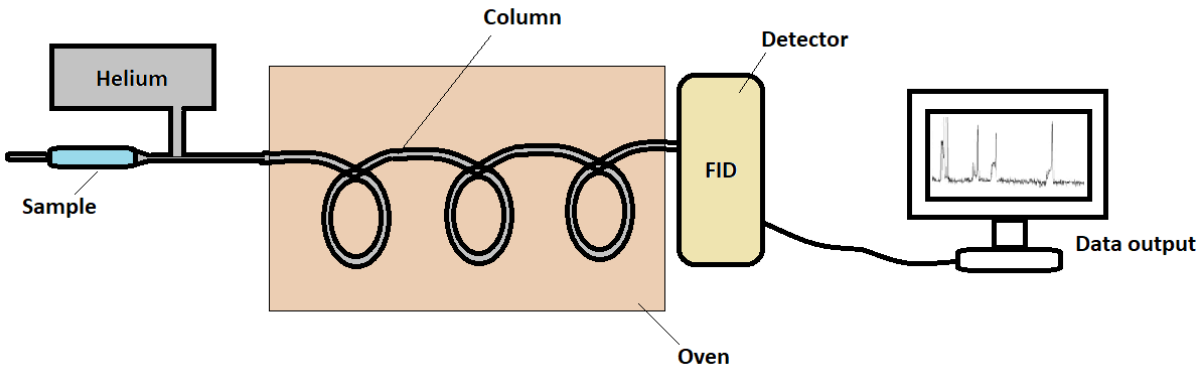

Figure 8 Illustration of the GC-FID system.

GC can be used to separate SCFAs in a sample solution. In the current project GC-FID was performed on faecal samples collected from mice bred in lab cages and pens. The GC-FID analysis was performed by engineers at a specified lab at NMBU. The protocol, reactants and instruments were provided by this lab.

2.2.1 Sample preparation

The faeces was collected from 20 pen mice and 24 lab mice aged 10 weeks, and flash-frozen before they were stored at -80°C until analysis.

The faecal samples were thawed, before solved in water with a specific dilution suited for the column in the GC instrument. The samples were then homogenized for 2 x 40 seconds at 1800 rpm., using Fastprep (MP Biomedicals). After the homogenization the sample solutions were gently spun down, to collect the larger particles. 300 µl of the supernatant was collected and transferred to a clean tube. 1:1 vol/vol of 1000 uM 2-methylvaleric acid was added as an internal standard. Further on, the sample solutions were centrifuged at 13 000 rpm., for 10 minutes, before 300 µl of the supernatant was transferred to 0,2 µm filtered spin columns and centrifuged again for 5 minutes at 10 000 rpm. The preparation of the samples was complete, and the solutions were transferred to GC vials, ready to be injected into the GC instrument.

2.2.2 GC analysis

The GC was performed with a Trace 1310 (ThermoFisher scientific) instrument, with Helium as the carrier gas. The injection volume of each sample was 0,2 µl, with a 2,5 ml/min flow speed through the column. Each sample was exposed to a number of different of temperatures from 90°C to 245°C, for about 15 min, to find the boiling point of each SCFA. The detector used was a FID.

Table 5 Showing the boiling point of each SCFA, detected in the GC. In addition to the Acid dissociation constant (Ka).

Acid	pKa	Boiling point (°C)
Formic acid	3.77	100,8
Acetic acid	4.76	118,1
Propionic acid	4.87	141,2
Isobutyric acid	4.87	155,0
Butyric acid	4.82	163,5
Isovaleric acid	4.98	176,5
Valeric acid	4.82	186,0
2-methylvaleric acid	4.84	196-197

2.2.3 Processing of GC-FID data

The data output produced by the GC is a chromatogram showing detected signal over time, as each compound has been carried through the column and the FID. A chromatogram consists of peaks which represent the observations of each compound of the sample. Peak height and width can be calculated, which is used to calculate concentrations and calibrate curves of samples with unknown composition. These calculations were done by the engineers performing the GC-FID analysis, providing concentrations (µM) of each SCFA in each individual sample.

The relative concentration results from the GC was normalized using the internal standard *2-methylvaleric acid*. The internal standard has a known concentration and is added in every sample that is analysed. In the calibration curve, the internal standard is used to compare peak areas of the data output. The internal standard is also used to improve precision of the analysis.

Multiplying the results normalized using the internal standard with the dilution factor of each individual sample, results in the amount of SCFA in the original sample.

2.3 Statistical analyses

The statistical analyses was conducted using the *GraphPad Prism 8* software. For each dataset of results, the most accurate statistical model was chosen, considering if the results were normally distributed or not. To determine if the results was normally distributed, multiple normality tests was conducted; *Anderson-Darling test*, *D'Agostino & Pearson test* and *Shapiro-Wilk test* was performed, using GraphPad Prism 8.

For all of the statistical methods used, the level of statistical significance was set to 0.05. This level, represents a measurement of the probability of making the observations one did, assuming that a null hypothesis is correct (Greenland et al., 2016). The results presented is analysed either as average of a group or the median of a group, with 95% confidence interval (CI) or interquartile range (IQR). Which statistical model used for each analysis is described by the figures in the result chapter.

3.0 Results

3.1 Gene expression

Analysis of gene expression in mice colons was conducted using methods such as isolation of RNA from colon tissue, quality and quantification analysis and quantitative PCR. The goal was to see if there were any differences in expression of genes associated with gut health and homeostasis, between the two groups; lab and pen mice.

3.1.2 RNA quality and quantification

The quantity and quality of total RNA isolated from distal colon were measured using Nanodrop 2000 and Bioanalyzer. Where Nanodrop provided RNA concentrations (ng/µl) and purity measurements (260/280, 260/230), and bioanalyzer provided bond-like images and corresponding electropherograms with detection of 18S- and 28S ribosomal RNA (rRNA), presented by peaks.

The results of the quality and quantification analyses showed on average total RNA of good quality and with mostly high concentrations, though with a few deviants.

4.2.1 Quantitative PCR, expression of TNFα and iNOS was significantly higher in pen mice

To compare the impact of a more natural and “dirty” environment (Pen mice) with semi-sterile lab mice (Lab mice), we assessed the relative mRNA levels of *iNOS, NOD1, NOX1, NOX2, MUC2* and *TNFα* from colonic mucosal tissue. As shown in figure 9, *iNOS* and *TNFα* was significantly different between the two groups, indicating that pen mice are exposed to stimuli that in fact stimulate to a weak inflammation. All the other genes also appeared to have higher mRNA levels in pen mice, but this is only a tendency with p-values ranging from 0.18 (NOD1) to 0.40 (NOX1).

The expression data for *TNFα, NOD1* and *NOX2* were normally distributed, and student t-test was conducted to determine significant differences and p-values. TNFα had a p-value of 0.0491 (significant), *NOD1* had a p-value of 0.1816 (not significant) and *NOX2* had a p-value of 0.1751 (not significant). Expression data for *iNOS, NOX1* and *MUC2* were not normally distributed, and were analysed using Mann-Whitney test. The p-value for iNOS was 0.0279 (significant), for *NOX1* it was 0.4002 (not significant) and for *MUC2* the p-value was 0.2428. As demonstrated in the plots presented below (figure 9), results from all genes showed 1-3 outliers with higher expression than other values of each plot, which might complicate statistical analyses determining significant differences.

Comparing the different genes within each group, the distribution of expression levels was the same in both lab- and pen mice. Showing that *MUC2* clearly had the highest expression level, followed by*; NOD1, NOX1, NOX2, TNFα* and *iNOS*.

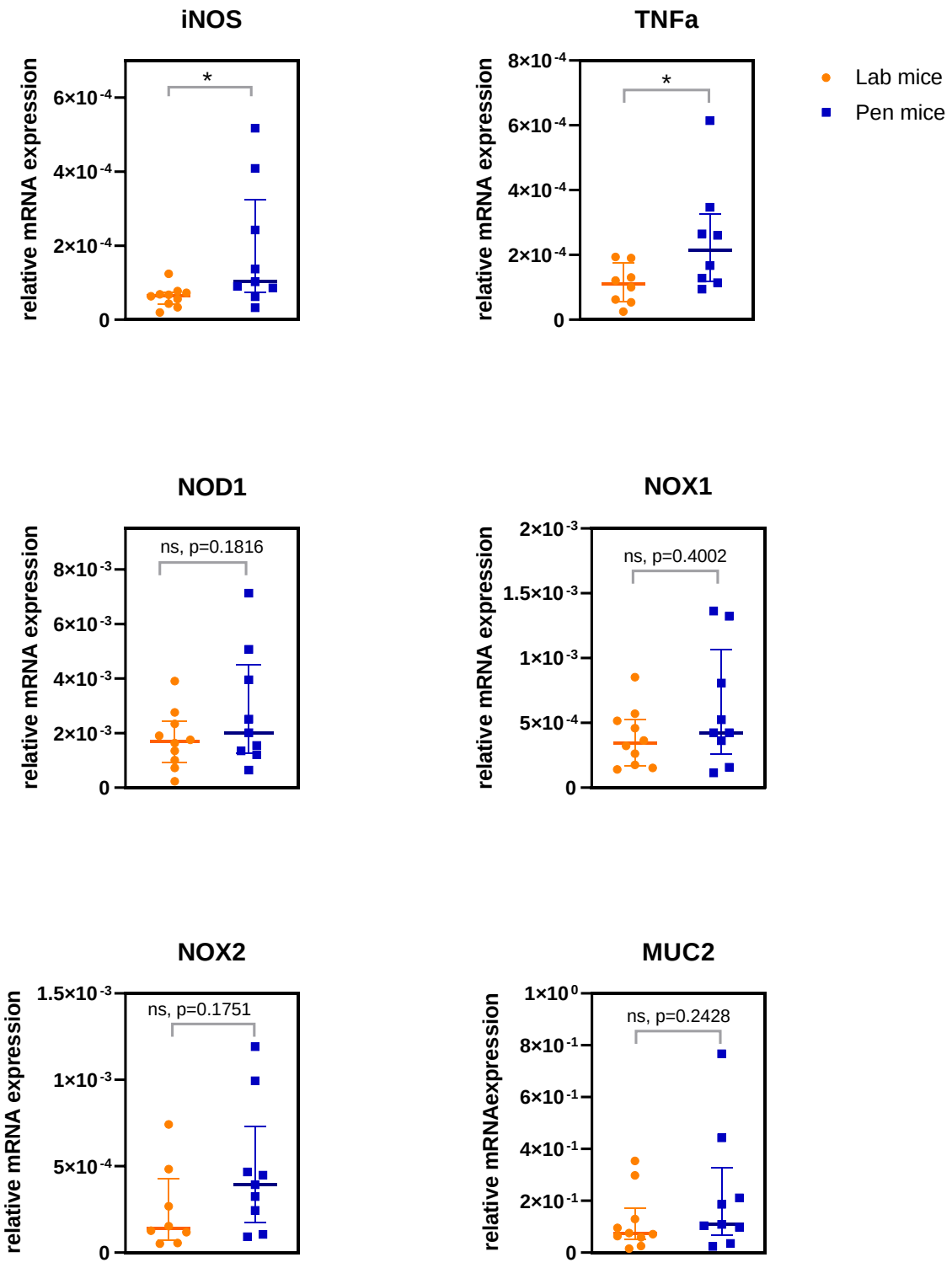

*Figure 9 Relative gene expression of iNOS, TNFα, NOD1, NOX1, NOX2 and MUC2 in colonic mucosa, comparing lab- and pen mice. Demonstrating a significant difference in expression of iNOS and TNFα between lab- and pen mice. The results from TNFα and NOD1 were normally distributed and statistically analysed used students t-test . iNOS, NOX1, NOX2 and MUC2 were not normally distributed and were statistically analysed using Mann-Whitney test, all expression data are presented with median and IQR. (Significant difference level p=0.05), ns; not significant, *p≤0.05. (iNOS n=9 pen mice, 10 lab mice. TNFα n=8 pen mice, 9 lab mice. NOD1 n=9 pen mice, 10 lab mice. NOX1 n=9 pen mice, 10 lab mice. NOX2 n=9 pen mice, 8 lab mice. MUC2 n=9 pen mice, 10 lab mice)*

3.2 SCFA analysis

To study the levels of faecal SCFAs, mainly focusing on butyrate, and its putative impact on gene expression, a GC-FID analysis was conducted on faeces collected from 24 lab mice and 20 pen mice.

3.2.1 SCFA content in lab- and pen mice, significant higher concentration of acetate and butyrate in lab mice (A)

Using the median of each SCFA concentration (µM) within each group of mice, the faecal level of each SCFA was calculated, the results is presented with 95% confidence interval (CI). None of the SCFA concentration results were normally distributed, and Mann-Whitney tests were conducted on all datasets to determine significant differences and p-values.

In lab mice faeces, butyric acid (butyrate) was the SCFA with the second highest concentration, in pen mice it had the third highest concentration. There was also a significant difference in faecal butyrate level between the two groups, with a p-value of 0.0002. The concentration of acetic acid (acetate) in faeces exceeded all the other SCFA levels, in both lab- and pen mice and showed a significant difference between the two groups, with a p-value of 0.0002. The faecal propionic acid (propionate) concentration was the second highest in pen mice, and third highest in lab mice, showing no significant difference between the two groups, with a p-value of 0.3555. The other three SCFAs; Valeric acid, isobutyric acid and isovaleric acid had lower and approximately the same concentrations in both groups, showing no significant difference between the two groups, with p-values >0.05. These results is presented in plot A, below.

3.3.2 Higher concentration of butyrate in lab mice (B)

The main aim of the GC-FID analysis was to determine if there was any difference in faecal butyrate level between the two groups; lab- and pen mice.

The significant difference (p=0.0002) in faecal butyrate concentrations between lab- and pen mice was determined using a Mann-Whitney test, showing a higher concentration in faeces from lab mice than in faeces from pen mice. The results in plot B is presented as individual values, median and IQR. The individual values of lab mice vary a lot and are widely spread in the plot, whereas the values from pen mice are all more similar as shown in the plot.

3.3.3 Percentage of each SCFA in the total content, higher percentage of butyrate in lab mice (C,D)

Using the means of each SCFA concentration within each group of mice, a part of whole-analysis calculated the distribution of each SCFA in the mice faeces. This distribution provides information about the amount of each SCFA in relation to the total amount of SCFAs.

Acetate clearly represented the largest part of the total SCFA content with approximately 60% out of 100%, in both lab- and pen mice. In lab mice faeces, butyrate made up the second largest part of the total content, with about 20%. In pen mice faeces, butyrate represented about 10% of the total SCFA content, which was a smaller part than in lab mice. Propionate represented approximately 13% of the content in pen mice faeces, while it represented a smaller part in lab mice, with approximately 9%. The remaining percent of the total SCFA content was evenly distributed between the three other SCFAs. The bars presented in plot C and D shows the percentage of each SCFA compared to the other SCFAs present, in both groups.

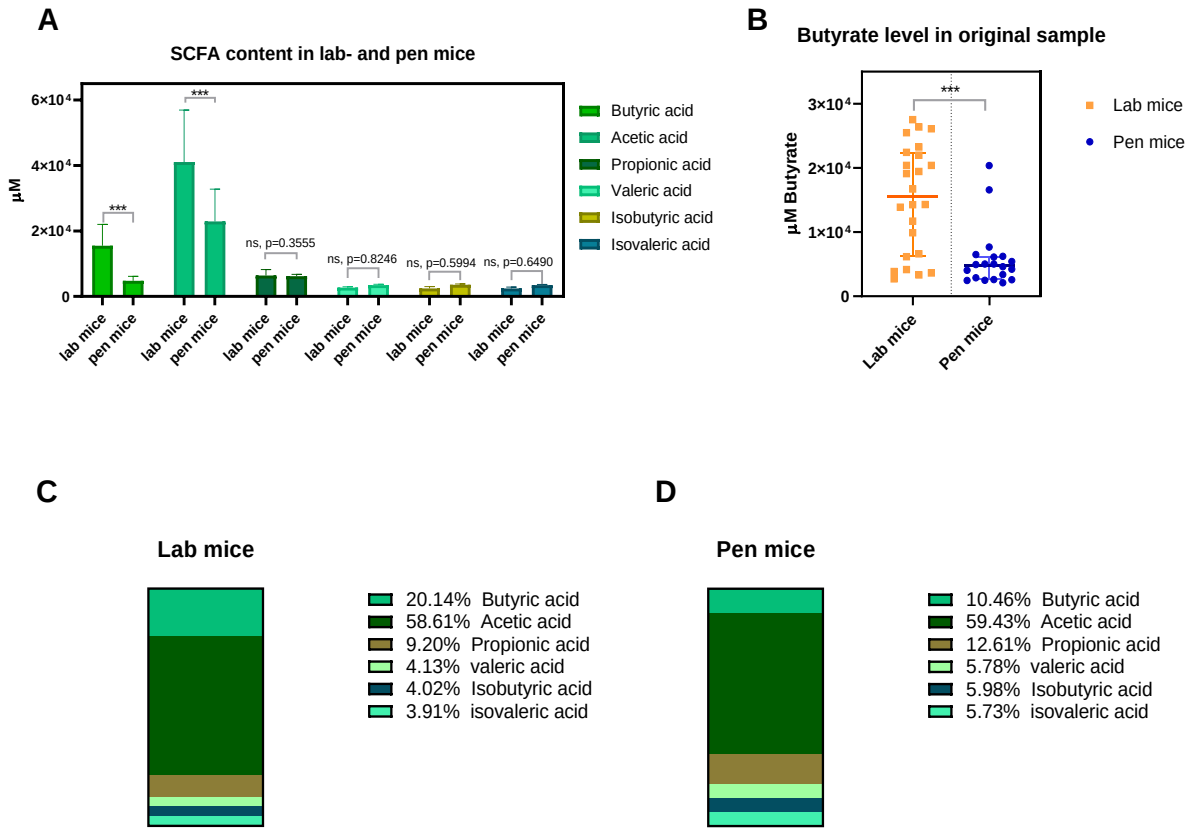

Figure 10 ***A:*** *Concentration (µM) of SCFAs; Butyric acid, Acetic acid, Propionic acid, Valeric acid, Isobutyric acid and Isovaleric acid comparing lab- and pen mice faeces. The presented results is the median shown with 95% CI. Statistical significance between groups were determined by Mann-Whitney test, which show significant difference in Butyric acid and acetic acid concentrations between lab- and pen mice, and no significant difference in the other SCFAs between the two groups. (Significant difference level p=0.05), ns; not significant, ***p≤0.01.* ***B:*** *Comparing concentration (µM) of faecal butyrate between lab- and pen mice, showing individual measurements and median with interquartile range (IQR). The comparison showed a significant difference in faecal butyrate concentration between lab- and pen mice. (Significant difference level p=0.05), ***p≤0.01.* ***C:*** *Parts of whole analysis, demonstrating the percentage each of the SCFAs make up of the total content (100%) in Lab mice, using the means of each SCFA. Demonstrating Acetic acid (58.61%) to be the dominant SCFA of the total content, butyric acid to be the second largest part (20.14%) and propionic acid (9.20%) to be the third largest part. Valeric acid, isobutyric acid and isovaleric acid make up about the same part (3.91-4.13%) of the total content, as demonstrated. n= 20 pen mice, 24 lab mice*

4.0 Discussion

Intestinal bacterial colonization starts at early age and has profound effects on the physiology and development of host immune system, and thus also affects disease and health throughout life. Several studies have emphasized that gut microbiota and its metabolites, such as the SCFA butyrate, are important components in differentiation and proliferation of different type of cells, including immune cells and intestine epithelial cells, promoting gut health and promoting tissue homeostasis. One of the most striking examples of the microbiota for the development of the immune system, was presented in a study by Smith et al., where they observed germ-free mice, bred in the absence of microorganisms. These mice proved to have a underdeveloped immune system (K. Smith et al., 2007).

The aim of this project was to analyse mice faeces and colon tissue from lab mice and pen mice, to look at bacterial metabolites and genes expression associated with gut health and homeostasis. To compare the two groups and try to determine if mice bred in an enriched microbial environment (pen mice) had any advantage over mice bred in semi-sterile lab cages (lab mice) when it comes to protection against pathogens and maintaining colon health.

Throughout the project it was shown that expression of *TNFα* and *iNOS,* were significantly higher in mucosa from pen mice than from lab mice*. Expression of NOD1, NOX1, NOX2* and *MUC2* demonstrated a tendency to upregulation in pen mice, compared to lab mice, although the difference was not significant.

Butyric acid was the SCFA of choice to focus on, due to it being the main energy source for healthy epithelial cells and its effect on enzymes associated with immune response and epithelial barrier function. The GC-FID showed that there was significant higher concentration of butyrate and acetate in in lab mice faeces, than in pen mice faeces. The concentration of faecal propionate tended to be higher in lab mice faeces, although not significantly so. The concentration of faecal valeric acid, isobutyric acid and isovaleric acid tended to be higher in pen mice, than lab mice, but not significantly so.

Studies conducted on mice researching intestinal inflammation and disease development, often use a dextran sodium sulfate-model (DSS-model). Treating mice

with DSS causes intestinal inflammation, and is used to study the development of inflammation and host immune responses during inflammation (Chassaing et al., 2014). The lab- and pen mice in the present project, did not undergo any treatment other than the exposure to different environments, thus the colons and faeces collected from these mice was assumed to be healthy. The research carried out in this project is therefore aimed at studying mechanisms of promoting gut homeostasis and health in normal colons. Considering this, the gene expressions or faecal SCFA content were not expected to show very big differences between the two groups of mice, especially not expression of genes mainly expressed during inflammation.

Topics discussed further on in this chapter;

- The impact of exposure to an enriched microbial environment on expression of *iNOS, NOD1, NOX1, NOX2, TNFα* and *MUC2*
- The impact of exposure to an enriched microbial environment on faecal SCFA concentration, and lower faecal butyrate levels in pen mice, due to oxidation of butyrate
- Regulation of gene expression due to the presence of butyrate.
- Methodical considerations

4.1 The impact of exposure to an enriched bacterial environment on expression of *iNOS*, *NOD1, NOX1, NOX2, TNFα* and *MUC2*

Commensal gut microbiota contributes to intestinal homeostasis by inducing transcription of genes associated with immune response and intestinal tissue health. Many of these genes, encodes enzymes which induces differentiation and proliferation, which leads to production of immune cells and epithelial cells. Renewal of colonic epithelia and immune cells, and controlling the differentiation of these, helps strengthen the protective mucosal barrier function of colon epithelia. Knowing this, one can assume that an intestine with a highly developed and diverse microbiota, would have a highly developed immune system as well. Based on this, it is interesting to research the expression of genes in colonic mucosa, associated with intestinal health and homeostasis.

iNOS, NOD1 and *TNFα* are all involved in the NF-κB pathway, either as activators triggering the NF-κB or as products of NF-κB induced gene transcription. Regulation of NF-κB has shown to be important in maintenance of the balance between pro-inflammatory and anti-inflammatory activity, due to the NF-κB pathway inducing cytokine production and gene transcription.

The relative expression of *iNOS* was significantly upregulated in pen mice, compared to lab mice. *iNOS* is mostly expressed in acute inflammation, which might be the reason for it being one of the genes overall least expressed in the mucosa in the present project, as there is no known inflammation in colons collected from pen- nor lab mice. However, expression of *iNOS* can also occur without inflammation, by LPS of bacteria activating signaling pathways which induces transcription. Since LPS is a cell wall compound of gram negative bacteria and assuming pen mice had a more developed microbiota than lab mice, it would be expected that pen mice also would have had higher levels of LPS, which lead to transcription of pro-inflammatory cytokines, such as iNOS. iNOS and other pro-inflammatory cytokine are important contributors to maintaining the balance of pro-/anti-inflammatory responses in the gut. Thus, making sure the gut immune system is primed and active, but not overly active leading to inflammation diseases.

the enzyme iNOS, produces nitric oxide (NO), which is shown to be an important signal molecule. Activated macrophages can inhibit the replication of pathogens

recognized by PRRs, by secreting effector molecules, such as NO. Significantly upregulation of iNOS in pen mice, compared to lab mice might indicate that the production of NO was also higher in pen mice. In literature, in has been a lot of debating whether iNOS and NO has beneficial or damaging effects on colon health, and whether *iNOS* is expressed in healthy colon as well as inflamed colons. A study performed by P. Roberts, et al. shows that iNOS is expressed in normal uninflamed human colons, suggesting that NO produced by iNOS may act as an oxidative barrier possibly by creating peroxynitrite by the reaction with super oxide produced by NOX1, reducing bacterial translocation and providing a means of defence against pathogenic microorganisms (Roberts et al., 2001). Assumed high diversity in microbiota in pen mice compared to lab mice, one could expect that there would also be higher levels of LPS inducing the transcription of *iNOS* and thus the production of NO.

TNFα relative expression in pen mice mucosa was significantly higher than in lab mice mucosa. TNFα, is a pro-inflammatory cytokine mainly produced by macrophages and facilitates for activation and translocation of NF-κB , inducing gene transcription. Ruder et al., highlights the role of *TNFα* in promotion of colonic health and tissue homeostasis, by activating other immune cells and mediating production of additional pro-inflammatory cytokines (Ruder et al., 2019). With the assumed exposure of pen mice to a vast load of microorganisms in their pens, compared to lab mice in semi-sterile cages, the mucosa in pen mice colons could be expected to have to deal with a higher number of bacterial antigens, leading to high activity of macrophages, constituting the first line of the immune system by recognition of pathogens. The putative high activity of macrophages, would explain the higher activity of TNFα in pen mice colons than in lab mice, due to TNFα being released by macrophages.

There was no significant difference in relative expression of *NOD1* in colonic mucosa between lab- and pen mice in the present project, although the results demonstrated a tendency of upregulation of *NOD1* in pen mice. *NOD1* encodes a PRR, which is known to be crucial in recognizing pathogenic agents such as bacterial peptidoglycan (PGs), which activates NF-κB leading to transcription of various cytokines associated with inflammatory immune response and maintenance of the inflammatory balance of the gut. Some studies also suggests that NOD1 activation of the NF-κB also has a

crucial role in maintaining the integrity of the colonic epithelium, through the adapter molecule NEMO/IKKγ. A study by Nenci et al., proved that inhibiting the NEMO activity of NF-κB in mice resulted in increased intestinal epithelial apoptosis and spontaneous colitis (Nenci et al., 2007). Another study, conducted by Clarke et al., showed that microbiota are a source of PGs that systematically primes the immune system, by binding to NOD1-receptors and activating NF-κB (Clarke et al., 2010). Assuming that pen mice had a more developed microbiota than lab mice, the expected outcome would be upregulation of *NOD1*, due to its ability to bind PGs of the microbiota.

Relative expression of *NOX1* showed a tendency of upregulation in colon from pen mice, although no significant difference compared to lab mice. The same for expression of *NOX2*; a tendency of upregulation in pen mice, compared to lab mice, though not significantly different. *NOX1* was higher expressed than *NOX2*, in both groups. *NOX1* and *NOX2* are homologues, encoding ROS producing enzymes NOX1 and NOX2. ROS are reactive oxygen species, which if not regulated is mostly known to cause DNA, protein and tissue damage. However, more recent studies have shown that NOX1-induced ROS activity in the colon, mediates tissue homeostasis and cell renewal, in addition to production of pro-inflammatory cytokines and proliferation of normal intestine epithelial cells (Burtenshaw et al., 2017). Health-beneficial NOX2 activity in the colon is not as well known, but some studies suggest that NOX2 activity in the gut is important in bacterial phagocytosis during inflammation. Ramonaite et al., showed that bacterial products, such as LPS, cooperating with pro-inflammatory cytokines, such as TNFα, have the ability to stimulate the *NOX*-expression and NOX induced ROS production in colon epithelial cells (Ramonaite et al., 2014). There was no significant difference in the expression of *NOX1* or *NOX2*, but the tendency of upregulation of both genes in pen mice mucosa, could be explained by the high expression of *TNFα* in pen mice. If so, it would support the theory of pen mice having a developed microbiota, due to TNFα cooperation with bacterial LPS.

Relative expression of *MUC2* showed to have the overall highest expression of all genes in this project, both in pen mice and lab mice. Naturally so, due to the encoded protein of this gene being a mucin, making up the protective mucus coating the colonic mucosa. Mucin-glycans are an important energy source for commensal

bacteria of the gut, the microbiota. Fermenting bacteria of the gut uses this energy to produce SCFA by bacterial fermenting, which can diffuse through the mucus layer of the intestine and be used as an energy source for colonocytes. The difference in expression of *MUC2* between lab- and pen mice mucosa was not significant, although the expression results demonstrated a tendency of upregulation in pen mice. MUC2 proteins are the major mucin protein of the colonic mucus and is constitutively expressed in the colonic mucosa, which might explain why the relative expression of *MUC2* is not significantly different in the two groups. Although, the tendency of upregulation of *MUC2* in pen mice colon, might still demonstrate the differences in the protective properties of the mucus between the two groups.

Upregulation of *MUC2* can translate to a high number of MUC2 proteins in the colon epithelia, which indicates an intact mucus layer. A well-developed mucosal barrier coated with mucus in colonic epithelia, consist of a high level of Mucins (Linden et al., 2008). MUC2 has a very important role in maintaining tissue homeostasis and health, due to mucin glycans providing energy for gut bacteria with the ability to produce butyrate through bacterial fermentation, and butyrate being the main energy source for healthy colonocytes. In addition to indirectly providing energy for colonocytes, MUC2 makes up the protective mucus layer in the colon epithelia, protecting against invasive pathogens and cytokine induced tissue damaging. Van der Sluis et al., demonstrated that mice deficient of *MUC2* expression spontaneously developed colitis (colonic inflammation), suggesting that the colonic mucosal layer, protected by mucins, is important in maintenance of colonic homeostasis and protection from external damage (Van der Sluis et al., 2006).

Studies conducted on mice researching intestinal inflammation and disease development, often use a dextran sodium sulfate-model (DSS-model). Treating mice with DSS causes intestinal inflammation, and is used to study the development of inflammation and host immune responses during inflammation (Chassaing et al., 2014). The lab- and pen mice in the present project, did not undergo any treatment other than the exposure to different environments, thus the colons collected from these mice was assumed to be healthy colons. Considering this, it might explain why relative expression of *NOD1, NOX1, NOX2* and *MUC2* was not significantly different between the two groups.

4.2 The impact of exposure to an enriched microbial environment on faecal SCFA concentration, and lower faecal butyrate levels in pen mice, due to oxidation of butyrate

Studies has shown that acetate, propionate, and mainly butyrate have beneficial effects on colon health, by maintaining metabolic homeostasis in colonocytes, induce anti-inflammatory activity and protect colonocytes from external damage (van der Beek et al., 2017). In a healthy colon, it is of high importance that colonocytes are protected against pathogenic agents and external damage, to be able to proliferate healthy cells in a normal manner and maintain tissue homeostasis. With butyrate being the primary energy source for healthy colonocytes (Parada Venegas et al., 2019), it plays an important part in protection of colonocytes and colonic mucosa.

GC-FID results showed that the average faecal butyrate concentration in pen mice was significantly lower than in lab mice. If this faecal concentration is used as an approximation of butyrate levels in the colon in vivo, as done in the study by D. Venegas et al*.,* (Parada Venegas et al., 2019), this questions the idea that pen mice have better gut health than lab mice, knowing that high butyrate levels in the colon promotes gut health. However, according to a study by Roediger, colonocyte oxidation of butyrate accounted for more than 70% of the oxygen consumption in human colon biopsy samples, which indicates that colonocytes rely on butyrate oxidation as their primary source, rather than glucose (Roediger, 1980). This could mean that lower levels of faecal butyrate might indicate a healthy colon after all, due to a higher consumption of butyrate as an energy source for colonocytes in the colon. Donohue er al., proved that colonocytes of germ-free mice have a deficit in mitochondrial respiration and undergo autophagy. They added butyrate in vitro to isolated colonocytes of germ-free mice, which resulted in retaining of both respiration and suppressing of autophagy (Donohoe et al., 2011).

4.3 Regulation of gene expression due to the presence of butyrate

Butyrate is known to effect gene expression, by upregulating expression of genes associated with maintenance of colonic mucosa and barrier function, in addition to being able to downregulate expression of genes associated with oxidative stress and proliferation of pathogens (Vanhoutvin et al., 2009). Butyrate is shown to inhibit the activation of NF-κB pathway, which regulates the production of pro-inflammatory cytokine such as TNFα and iNOS, and anti-inflammatory cytokines (C. Lee et al., 2017). Butyrate is also known to be a histone deacetylase inhibitor molecule, inducing anti-inflammatory activity by inhibiting gene transcription (Steliou et al., 2012). Based on this, it is interesting to research the differences in expression of some genes known to be associated with butyrate activity in the colon.

As mentioned, the transcription factor NF-κB , plays an important role in regulating the balance of inflammatory immune responses, by inducing transcription of genes, including inflammatory cytokines. According to Nancey et al., TNFα can inhibit butyrate oxidation in normal colonic mucosa, supporting the role of inflammatory cytokines in regulation SCFA uptake in colonic mucosa. Reduced butyrate oxidation results in a decreased energy supply to colonocytes, and may explain mucosal damage occurring during inflammation (Nancey et al., 2005). In a study by Segain et al., they investigated if butyrate reduces inflammation in the colon through inhibiting NF-κB and pro-inflammatory cytokine production. Where they proved, using a colon biopsy, that in the presence of butyrate, *TNFα* levels decreased significantly both in healthy and inflamed colon (Segain et al., 2000). Ruder et al., proved that patients suffering from colonic inflammation showed higher expression levels of *TNFα*, due to elevated numbers of TNFα -secreting immune cells (macrophages) in the colonic tissue (Ruder et al., 2019). If so, it indicates that individuals with a highly developed microbiota and therefore also an increased level of immune cells in colonic epithelia, could have increased activity of the pro-inflammatory cytokine TNFα. High excess levels of butyrate in the lab mice colons, might increasingly inhibit NF-κB transcription of inflammatory cytokines, including *TNFα and iNOS.*

iNOS and *NOD1* are associated with the NF-κB pathway as well. The NOD1 receptor activates the NF-κB pathway by recognizing and binding bacterial PGs, which induced transcription of several genes, including *iNOS,* initiating anti-microbial

immune response. In both pen mice colons and lab mice colons, *NOD1* expression is clearly more expressed than *iNOS*, suggesting that NOD1 receptors is recognizing bacterial PGs, but the NF-κB -induced iNOS transcription might be inhibited by butyrate. However, *NOD1* expression is still higher in pen mice than in lab mice, though not significantly different, but it might indicate elevated bacterial content in pen mice colons.

NOX1 and NOX2 are Reactive oxygen species (ROS) producers. If ROS activity is beneficial or damaging for gut health, has been debated in many studies. According to Rosignoli et al., butyrate can have so-called oxidant-activity, reducing oxidative stress caused by overproduction of ROS (Rosignoli et al., 2001). The mechanism of butyrate reducing oxidative stress is not well studied, even though several studies show that butyrate is able to beneficially effect oxidative stress in healthy colon (El-Shorbagy, 2017). If so, it would indicate that in the present project, lab mice would have less chance of developing oxidative stress in the colon, due to high levels of faecal butyrate. Unless, the mechanism of which butyrate reduces oxidative stress, results in degradation of the butyrate molecule, resulting in lower faecal levels as in pen mice.

MUC2 is an important protein making up the colonic mucosal layer, protecting the host from pathogenic agents of the gut, in addition to maintaining tissue metabolic homeostasis and functioning as an energy source for SCFA fermenting bacteria of the microbiota. Butyrate is fermented by gut bacteria and its energy, harvested from mucin glycans. Pen mice with assumed developed microbiota, expressed higher levels of *MUC2* than lab mice, which supports pen mice having an intact mucosal barrier in the colon. An intact mucosal barrier sustains tissue homeostasis, which again facilitates colonocyte-oxidation of butyrate, leading to a healthy gut epithelia. This chain of energy trading, also supports the theory of increased butyrate oxidation in pen mice, explaining their lower levels of faecal butyrate.

Inhibiting and/or inducing gene expression through NF-κB activity, regulating the balance of inflammatory immune responses and strengthening the mucosal barrier, among other mentioned mechanisms of butyrate, are all important in promoting gut health and tissue homeostasis.

4.4 Methodical considerations

4.4.1 RNA quality and quantification

Before synthesizing cDNA from total isolated RNA, NanoDrop and Bioanalyzer was used to determine concentration and quality of purified RNA from distal colon mucosa (samples #41-60). Being aware of the quality and amount of RNA before using the samples in further analyses is important, because the protocol and instrument used for PCR to synthesize cDNA has a recommended lower and upper concentration limit of RNA, to perform an optimized synthesis. Knowing the concentration of total RNA in the isolated samples made it possible to make working solutions with optimal RNA concentrations to use in the cDNA synthesis. In addition to being able to optimize the PCR reaction, it is beneficial to have knowledge about the RNA quality, if the RNA is intact or partly degraded, because it can explain possible deviations in gene expression analyses later on.

The results from the NanoDrop measurements showed that most total RNA samples had a concentration between 150-300 ng/µl, with some deviants with lower concentration below 100 ng/µl and some with higher concentration, over 400 ng/µl. Over all, this was more than enough RNA to conduct the cDNA synthesis PCR. The quality measurements provided from the NanoDrop showed that on average the total RNA was pure and of good quality, according to the 260/280 ratios, the 260/230 ratios was not as optimal. Most samples had 260/280 values between 2.0 – 2.2, which indicates high purity with minimal protein contamination. Some of the 260/230 values was measured below 2.0, and some even below 1.0, which show some deviation from the optimal value of 2.1, this deviation may indicate contamination of salt compounds, for example from the lysis buffer used in RNA isolation.

With the average high concentrations of total RNA isolated, it was possible to make working solutions prior to the cDNA synthesis, with concentrations of 100 and 200 ng/µl and a volume of 50µl. For the RNA samples with a measured (NanoDrop) concentration below 100 ng/µl, the total volume of the working solutions was scaled down to 20 µl, to fit the optimal concentration of 100 ng/µl.

In this project a total RNA amount of 0,8µg was added in the PCR mix used for cDNA synthesis, varying the volume of added working solution based on the

concentrations, 8µl (100ng/µl) or 4 µl (200 ng/µl) was added in the PCR mix. The iScript cDNA synthesis kit (Bio-Rad) used in this project can be used on total RNA amount as small as 100 fg (femtogram) and up to 1 µg, by up- or down-scaling the volume of H_2O. Meaning, the total RNA amount used for cDNA synthesis in this project was adequate.

A Bioanalyzer analysis was conducted to look at RNA quality, and to determine if the RNA molecules were intact, or if they had started to degrade, or were already degraded. The Bioanalyzer software, normally holds a function that calculates values of RNA integrity (RIN), which provides detailed information about degradation of RNA molecules. The RIN calculation function of the software was defect when used in this project, therefore RIN values was not available. However, electropherograms was produced by the software, which also provides information about RNA quality. The Bioanalyzer detected fluorescens from certain structural ribosomal RNAs (18S and 28S ratios) of RNA molecules, and marked them as peaks in an electropherograms, one for each RNA sample. From this one can draw assumptions on RNA quality. An ideal electropherogram showing intact RNA should have high detection peaks of both 18S and 28S, where 28S is slight higher than 18S. 28S rRNA degrades easier than 18S rRNA, which means that if the detection peak of 28S is lower than 18S, it indicates that degradation of the RNA molecule has begun.

Bioanalyzer results from this project showed that the electropherograms from most of the total RNA samples, showed detection of fluorescens from both 18S and 28S ratios, indicating intact RNA. The electropherogram of RNA sample 55 shows both peaks, however the 18S peak is slightly higher than the 28S peak, which might indicate some degradation. The electropherogram of sample 59 was the only one with only one detected peak, where the 28S rRNA ratio was not detected. As an average summary, the electropherograms show total RNA of good quality, with minimal degradation.

4.4.2 Gene expression analyses

After synthesis of cDNA, relative expression of iNOS, NOD1, NOX1, NOX2, TNFα and MUC2 was measured, using qPCR. The expression data was normalized using a reference gene GAPDH, whose expression was assumed to be stable between individuals, experimental conditions or physiological states. GAPDH is one of the most commonly used housekeeping genes, used in comparisons and normalizations of gene expression data (Robert D Barber et al., 2005). A critical step in the RT-qPCR workflow for studying gene expression is data normalization, one of the strategies being the use of reference genes (Llanos et al., 2015). Studying the amplification curve from the reference gene will determine that the combinations of reaction reagents and cDNA templates are ideal and intact, demonstrating that fluorescens can be detected (Pabinger et al., 2014).

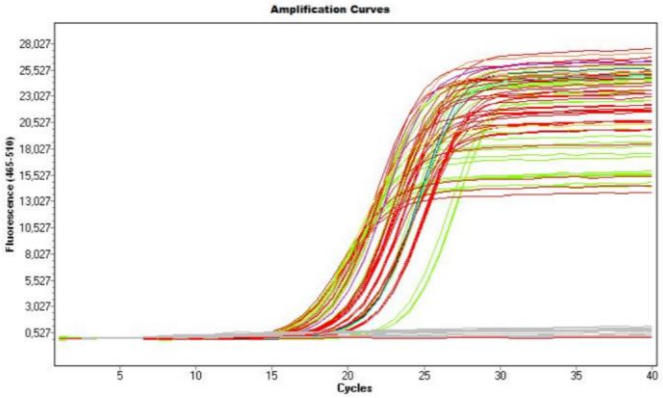

Figure 11 Amplification curves of the reference gene GAPDH from the qPCR run in present project, showing stable detection of fluorescence and Cq values ~15-20, for all samples. NAC and NTC have no detected fluorescence and are demonstrated as flat curves.

4.4.3 SCFA analysis

The SCFA content in mice faeces was measured using GC-FID. The analysis was performed by engineers at a specified lab at NMBU. The processing of data and calculating the concentrations of each SCFA was done by the engineers performing the GC-FID analysis, providing concentrations (µM) of each SCFA in each individual sample. Since the analysis and the processing of data was carried out at the specified lab, one has to trust that the analysis was carried out according to the

protocol, and that the reagents provided from this lab were intact and combined to optimize the analysis.

The relative concentration results from the GC was normalized using the internal standard *2-methylvaleric acid*. The internal standard has a known concentration and is added in every sample that is analysed. In the calibration curve, the internal standard is used to compare peak areas of the data output. The internal standard is also used to improve precision of the analysis. The detection of the internal standard for all faeces samples as presented in the method chapter, indicates that the analysis and regents were intact and worked as planned.

5.0 Conclusion

Assesment of faeces and colons collected from mice, demonstrated that the effects of an enriched bacterial environment on pen mice resulted in lower faecal butyrate levels, compared to lab mice. This was unexpected, due to the putative more diverse microbiota in pen mice. Although the present project can provide indications only, and the difference in expression of most genes between lab- and pen mice were not significant, some conclusions on the beneficial effects of an enriched bacterial environment is suggested.

Relative expression of *TNFα* and *iNOS* was significantly higher in colonic mucosa from pen mice, than from lab mice. High expression of these genes indicates a developed microbiota, due to their association with bacterial cell wall components such as LPS, and that there are more or more potent macrophages in the pen mice colon, supported by TNFα mainly being secreted by macrophages.

These findings demonstrates that exposure to an enriched microbial environment facilitates for development of a diverse microbiota and the development of the immune system, leading to expression of genes related to maintenance of gut health and homeostasis such as *TNFα* and *iNOS*.

The tendency of upregulation of *MUC2* in colonic mucosa from pen mice, together with the same tendeny of *NOX1* and *NOX2* in pen mice, indicates a well-developed microbiota, in addition to high epithelial turn over rate, tissue homeostasis and intact mucosal barrier function. The lower level of faecal butyrate in pen mice, might be explained by the rapid turnover rate of colonic epithelia, and butyrate being the main energy source of colonic epithelia, leading to the oxidation of butyrate being increased as well. The tendency of upregulating of *NOD1* indicates a well-developed microbiota, due to its association with bacterial components such as PGs and LPS.

The presence of butyrate in the colon is important for maintenance of the balance of pro- and anti-inflammatory immune responses, by inhibiting NF-κB and regulating transcription of pro-inflammatory cytokines, such as iNOS and TNFα. This regulation makes sure the immune cells, such as macrophages, are primed and ready to act on pathogens, but are not overly active which may cause inflammation diseases.

Considering all the results together with the acquired knowledge about the collaborative relation between butyrate and the selected genes, it indicates that being exposed to an enriched bacterial environment strengthens the immune system and the mucosal barrier function, by facilitating for the development of gut microbiota and its metabolites.

6.0 Future perspectives

Further research on disease development and intestine health, using lab animals exposed to a bacterial environment is encouraged. It is important to highlight the importance of environmental effects on development of a diverse microbiota, with its beneficial effects on gut health. This approach is important for making the animal studies have more translational value to humans, and to get more reliable results when studying prevention of disease or disease development in the gut.

Further research in this field should keep focusing on the importance of butyrate and other microbiota metabolites in the gut, in addition to look at the expression of more genes associated with gut health in collaboration with these metabolites. Genes such as *TLR4* and *Interferon-γ (IFNγ),* which is associated with bacterial components such as LPS and the NF-κB pathway, would be interesting to study further. In addition to studying more genes associated with microbiota and its beneficial impact on host health, it would be interesting to do a protein assay as well, to determine if these encoding genes actually is translated. It would also be of interest to carry out assays to determine which microbes actually exist in the gut of these mice, to be able to research the development of microbiota and their metabolites more accurately.

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8.0 Appendices

8.1 Instruments, kits and materials

Table 6 Instruments

Instruments	Manufacturer
NanoDrop 2000c	NanoDrop Technologies, Wilmington, USA
Agilent 2100 Bioanalyzer	Agilent Technologies, Santa Clara, USA
LightCycler 480 Instrument II Roche	Applied Science, Indianapolis, USA
C1000 Thermal Cycler	Bio-Rad Laboratories

Table 7 Kits

Kits	Manufacturer
Agilent RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, USA
iScript cDNA Synthesis Kit	Bio-Rad Laboratories, Hercules, USA
NucleoSpin RNA/Protein Purification kit	Machery-Nagel, Düren, Germany

Table 8 Other reagents and materials

Reagents and materials	Manufacturer
5x HOT FIREPol EvaGreen qPCR Supermix	Solis BioDyne, Estland
LightCycler 480 Multiwell 96w Plate	Life science Roche
2-Mercaptoethanol, (βmercaptoetanol)	Sigma-Aldrich
RNAlater	Sigma-Aldrich

8.2 Software

Table 9 Software

Software	Reference
Nanodrop 2000/2000c Software	Thermo Scientific. Nanodrop 2000/2000c (Version; 1.6.198). Reference: https://www.thermofisher.com/no/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/ultraviolet-visible-visible-spectrophotometry-uv-vis-vis/uv-vis-vis-instruments/nanodrop-microvolume-spectrophotometers/nanodrop-software-download.html
LightCycler® 480 Software	Idaho Technology. LightCycler 480 Software (version; 1.5.1.62). Reference: https://lifescience.roche.com/en_no/products/lightcycler14301-480-software-version-15.html
LinRegPCR January 2020	Ruijter, J. M. LinRegPCR (Version; 2020.0) Reference: https://www.medischebiologie.nl/files/
GraphPad Prism 8	GraphPad Software Inc. GraphPad Prism 8 (Version; 8.42) Reference: https://www.graphpad.com/scientific-software/prism/
2100 Expert Software	Agilent Technologies. 2100 Expert Software (Version B.02.07.SI532). Reference: https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-software
NCBI Primer-BLAST tool	Reference: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

8.3 Manufacturer´s manuals

Table 10 Manuals

Manuals	Manufacturer and reference
RNA and protein purification User manual NucleoSpin® RNA/Protein	Macherey-Nagel, March 2019 / Rev. 10 Reference: https://www.mn-net.com/Bioanalysis-Downloads Read: 07.05.2020
NanoDrop 2000/2000c Spectrophotometer V1.0 User Manual	Thermofisher Scientific, 2009, V1.0, Reference: https://assets.thermofisher.com/TFS-Assets/CAD/manuals/NanoDrop-2000-User-Manual-EN.pdf Read: 23.04.2020
Agilent RNA 6000 Nano Kit Guide	Agilent. Edition; 07/2013. Reference: https://www.agilent.com/cs/library/usermanuals/Public/G2938-90034_RNA6000Nano_KG.pdf Read: 23.04.2020
Solis Biodyne	Solis-Biodyne; Reference: https://www.solisbiodyne.com/pics/8142_Data_Sheet_HOT_FIREPol_EvaGreen_qPCR_Supermix.pdf Read: 15.05.2020

8.4 Primers

Table 11 Primers used in qPCR

Genes	Primers	Annealing temperature in qPCR, (°C)
iNOS	Forward GACATTACGACCCCTCCCAC Reverse ACTCTGAGGGCTGACACAAG	62
NOD1	Forward TGACAGTAATCTGGCTGACC Reverse GTCTGGTTCACTCTCAGCAT	59
TNFα	Forward CTGTCTACTGAACTTCGGGGTGAT Reverse GGTCTGGGCCATAGAACTGATG	61
NOX1	Forward GTGATTACCAAGGTTGTCATGC Reverse AAGCCTCGCTTCCTCATCTG	64
NOX2	Forward GGGAACTGGGCTGTGAATGA Reverse CAGTGCTGACCCAAGGAGTT	61
MUC2	Forward GATAGGTGGCAGACAGGAGA Reverse GCTGACGAGTGGTTGGTGAATG	63
GAPDH (HK)	Forward CTTCAACAGCAACTCCCACTCTT Reverse GCCGTATTCATTGTCATACCAGG	60

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