Dietary regulation of the signalling molecule FGF15 in the ileum and its effect on the gut-liver axis

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Abstract

The intestinal hormone Fibroblast Growth Factor 19 (FGF19, rodent homologue FGF15) has recently been linked to both positive and negative health outcomes. The expression of FGF15/19 is regulated by the bile acid-sensing transcription factor farnesoid X receptor in the ileum. After its production, this enterokine travels to the liver where it blocks *de novo* bile acid production via CYP7A1. Circulating FGF15/19 levels are known to rise postprandially which can influence both blood cholesterol and glucose levels. However, the influence of dietary patterns on the regulation of FGF15/19 expression is largely unknown.

In this thesis, dietary regulation of FGF15 is explored in rodent studies. Mice fed a high starch (HS) diet showed increased expression of FGF15 compared to mice fed a chow control diet. The importance of starch digestion was confirmed by acarbose treatment, which inhibited both starch digestion and FGF15 expression. Further, increased glucose in the ileum was shown to induce the expression of FGF15. Importantly, this regulation was found to be independent of the microbiota. In addition, in mice fed HS diets supplemented with soluble fibres, FGF15 expression was suppressed. This latter effect may also contribute to the known cholesterol-lowering effects of soluble fibres.
While the HS diet induced the expression of FGF15, this work highlights the long-term negative health effects of such a diet, showing dysregulation of the gut-liver axis in our mouse model that eventually led to increases in cell proliferation, and, in some mice, the development of hepatic carcinoma. Reducing HS diets in a caloric restriction (CR) model counteracted these negative health effects and in the long term downregulated the expression of FGF15. Decreased FGF15 expression correlated with a reduction in ileal bile acids in the CR model.

In conclusion, we showed that increased luminal glucose reaching the ileum, consumption of soluble fibre, and long-term CR influenced the expression of the important gut-hormone FGF15. This influence on its regulation may influence gut-liver health with a profound impact on systemic bile acids and cholesterol levels.

List of abbreviations

AH receptor	Aryl Hydrocarbon receptor
ALT	Alanine transaminase
ASBT	Apical sodium dependent bile acid transporter
AST	Aspartate transaminase
BAD	Bile Acid Diarrhoea
BCO1	Beta-Carotene Oxygenase 1
BSH	Bile salt hydrolase
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CR	Caloric restriction
CYP1A1	Cytochrome 1, subfamily A, polypeptide 1
CYP27A1	Sterol 27-hydroxylase
CYP7A1	Cholesterol 7 alpha-hydroxylase
CYP8B1	Sterol 12-alpha-hydroxylase
DCA	Deoxycholic acid
DIO	Diet induced obesity
En%	Energy percent
FABP6	Fatty acid binding protein 6
FGF15	Fibroblast growth factor 15
FGF19	Fibroblast growth factor 19
FGFR4	Fibroblast growth factor receptor 4
FITC	Fluorescein isothiocyanate
FXR	Factor farnesoid X receptor
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GI	Gastrointestinal
GLCA	Glycolithocholic acid
H&E staining	Haematoxylin and eosin staining

HDCA	Hyodeoxycholic acid
HF	High fat
HS	High starch
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
IDO1	Indoleamine 2,3-dioxigenase 1
IECs	Intestinal epithelial cells
LCA	Lithocholic acid
LC-MS	Liquid chromatography–mass spectrometry
LPS	Lipopolysaccharide
MeOH	Methanol
MRM	Multiple reaction monitoring
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NMR	Nuclear magnetic resonance
OG	Oral gavage
OSTα/β	Organic solute transporter alpha/ beta
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PRRs	Pattern recognition receptors
QIIME	Quantitative Insights into Microbial Ecology
Q-PCR	Quantitative polymerase chain reaction
SHP	Small heterodimer partner
SI	Small intestine
SIBO	Small intestinal bacterial overgrowth
T-a-MCA	Tauro-α-muricholic acid
T-b-MCA	Tauro-β-muricholic acid
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TLCA	Taurolithocholic acid

TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
UDCA	Ursodeoxycholic acid
UEA	University of East Anglia
VSG	Vertical sleeve gastrectomy
WUR	Wageningen University
α-MCA	α-Muricholic acid
β-MCA	β-Muricholic acid
ω-MCA	ω-Muricholic acid

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Chapter 1: General introduction

Thesis Britt Blokker

1.1 The small intestine

The small intestine is the longest part of the gastrointestinal (GI) tract and can roughly be divided in three parts; duodenum, jejunum and ileum. Enterocytes along the length of the small intestine have specialized functions dictated by their position (Welcome 2018). Enterocytes in the duodenum are specialized towards digestive processes, which is supported by digestive enzymes secreted from the pancreas and bile acids from the gallbladder and liver (Stuart 2011). Additionally, initial absorption of glucose starts via the action of enterocytes in the duodenum (Rolston 1999). In the jejunum, enterocytes are specialized in the uptake of nutrients, with the bulk of the absorption of dietary fat, carbohydrates and amino acids also taking place in this anatomical region. More towards the latter part of the small intestine, in the ileum, vitamin B12 and bile acids are absorbed via the enterocytes (Stuart 2011; Davis and Attie 2008). Importantly, food digestion mostly takes place in the duodenum and upper jejunum, exposing the intestine to a high concentration of monomeric nutrients, like mono-saccharides, fatty acid and amino acids (Stuart 2011). The different specializations of the enterocytes along the tract of the small intestine reveals its complexity and highly regulatory nature. Relevantly, the regulation of differentiation between jejunal and ileal enterocytes has been linked to the expression of the transcription factor GATA4. When GATA4 was expressed in ileal enterocytes they lost their the expression of genes involved in bile acid metabolism, which are an important identifier of ileal enterocytes (Thompson et al. 2017).

1.1.1 Intestinal epithelial cells

Intestinal epithelial cells are ordered into crypts (crypts of Lieberkühn) and villi. The complete intestinal epithelium is replaced every few days and this process is driven by the functions of multipotent intestinal stem cells that arise from the intestinal crypts. (Li and Clevers 2010). Active stem cells in the lower part of small intestinal crypts can be identified by the expression of LGR5 (Barker et al. 2007) and produce two mature cell lineages: absorptive cells (enterocytes) and secretory cells (Paneth, goblet, enteroendocrine and tuft cells). The most abundant cell type in the small intestine villi are the enterocytes which are responsible for nutrient and bile acid absorption and metabolism and hormone production (Bowcutt et al. 2014; Hou et al. 2017). The secretory cell types

are located throughout the crypt villus' structure. At the base of the crypts, Paneth cells secrete antimicrobial peptides and proteins that are essential for host-microbe interactions. Paneth cell markers include lysozyme LYZ1 and LYZ2 (Clevers and Bevins 2013). Goblet cells produce mucus and thereby are responsible for the single layer of mucus that lines the small intestine. The dominant gene responsible for mucus production in goblet cells in the small intestine is MUC2 (Birchenough et al. 2015). Enteroendocrine cells comprise over 10 different cell types that produce distinct hormones, such as glucagon like peptide 1 (GLP-1), serotonin and secretin. Enteroendocrine cells can be identified by the expression of the tight junction protein claudin-4 (CLDN4) (Nagatake et al. 2014). Tuft cells have a chemosensory function and are the sole producers of the cytokine IL-25 in small intestinal cells. In healthy conditions tufts cells account for <1% of all cells in the small intestine, however, this can increase to 15% during infection (von Moltke et al. 2015). Taken together, all these specialized small intestinal cells form a monolayer of epithelium that cover the entire small intestine providing physiological support and protection for the host. In addition to this monolayer the small intestine host a large amount of immune cells, mostly present in the lamina propria located below the IEC monolayer, including macrophages, dendritic cells and T-cells (Mowat and Agace 2014).

1.1.2 Intestinal barrier function

The small intestine is essential for the absorption of nutrients, but also acts as a barrier between the food consumed and the internal organs. The intestine is among the first organs to be exposed to external components we consume. Further, the intestinal barrier function is crucial in the symbiotic relationship with the gut microbiota. Loss of intestinal barrier function has been linked to GI diseases such as inflammatory bowel disease (IBD), but also to a number of extra-intestinal autoimmune and inflammatory diseases including multiple sclerosis and type 1 diabetes (Peterson and Artis 2014). The IECs are protected by the mucous layer and form a physical barrier between the lumen of the GI tract and the host. Adjacent IECs are connected by tight junctions, which are built up by transmembrane proteins such as occludins and claudins and cytosolic scaffold proteins like zonula occludins (Bischoff et al. 2014). Although most literature on intestinal permeability focusses on the colon, small intestinal permeability is increasingly attracting more interest.

For example, small intestinal permeability has been shown to be increased in humans diagnosed with non-alcoholic fatty liver disease (NAFLD) (Miele et al. 2009; Kwak et al. 2014). In the small intestinal epithelium, there is the added challenge of having a high cell turnover; this leads to consistent apoptotic and live cell extrusion which occurs to help maintain intestinal barrier structure and function (Eisenhoffer et al. 2012).

1.2 The gut microbiota

The microorganisms living in our GI tract, collectively called the microbiota, have been studied extensively, with over 12,900 papers published in the last five years (Cani 2018). While the microbiota is present throughout the gut and consists of bacteria, archaea, viruses, phages, yeast and fungi, the majority of these publications focused on bacterial differences in the colon and faeces. Changes in the intestinal microbiota have been linked to a large variety of metabolic diseases including obesity and diabetes (reviewed in (Janssen and Kersten 2015)).

Bacteria in the gut can influence host health via a variety of mechanisms, most importantly via their interaction with host cells and their secreted metabolites. Bacteria interaction with host cells initially occurs with cells of innate immune system via pattern recognition receptors (PRRs) like toll-like receptors (TLRs). These receptors can recognise pathogen-associated molecular patterns (PAMPs) from microorganisms, for example lipopolysaccharide (LPS) derived most prominently from gram-negative species and can induce immune responses. Upon recognition of PAMPs by PRRs on immune cells, immunoregulatory signals are transduced to the large body of immune cells present in the lamina propria (Mowat and Agace 2014). Importantly, bacterial interaction with host cells (and consequent immunomodulatory responses) is a two way interaction; the innate immune system can respond to bacteria by initiating the immune response, however, the gut microbiota can also have an effect on GI immune system regulation which can depend on microbial composition (Hooper and Macpherson 2010). This interaction between host and microbes alters not only the immune response but also host metabolism. For example, LPS, has been shown to be linked to increase weight gain and insulin resistance development on a high fat (HF) diet (Everard et al. 2014). Additionally, increased circulating levels of LPS have been identified in obesity and diabetic patients (Lassenius et al. 2011; Pussinen et al. 2011) and small intestinal infusions of obese subjects with the microbiota of a lean donor has shown to increase insulin sensitivity (Vrieze et al. 2012).

Outside their direct interaction with host cells, the microbiota also produces metabolites that can alter the wellbeing of the host. The large variety of metabolites produced by the

gut microbiota include secondary bile acids, trimethylamine-N-oxide (TMAO), neurotransmitters and short chain fatty acids (SCFAs). As an example of a metabolite that negatively influences host health, TMAO has been shown to be produced from choline and L-carnitine after conversion by both the gut microbiota and hepatic enzymes and has been linked to an increased risk of atherosclerosis (Koeth et al. 2013). Conversely, the production of SCFAs (acetate, propionate and butyrate) from non-digestible carbohydrates by the microbiota has been linked to a number of positive health effects (Makki et al. 2018). Additionally, the SCFA butyrate has long been known as an important energy source for colonocytes, however recently it has been shown that butyrate can also influence oxygen consumption in the colon and thereby protect against the colonization of potentially pathogenic bacteria (Byndloss et al. 2017; Cani 2017).

Despite the large number of publications surrounding the gut microbiota, and the enormous progress made in the last years, a large part of the current evidence is correlative and additional research will be required in the coming years to establish causal effects (Cani 2018).

1.2.1 The small intestinal microbiota

The majority of scientific publications have focused on the microbiota in the colon and in excreted faeces which is mostly due to the ease and accessibility of these materials in humans. However, the major drawback of using faecal samples for microbiota analysis is that it only represents the excreted microbiota, leaving a large part of the microbiota unexplored. In more recent studies, the small intestinal microbiota has been explored and has been shown to be of importance for host health (El Aidy, van den Bogert, and Kleerebezem 2015). Comparatively with microbial populations in the colon, the microbiota in the small intestine is lower in both numbers and diversity and is closer in proximity to the host epithelium (and thereby underlying immune cells). The latter is due to a single thin mucus layer found in the small intestine (Mowat and Agace 2014) compared to the large intestine which comprises a thick mucus layer where bacteria reside at relatively further distances from host epithelial tissue. Importantly, close contact between the bacteria and the small intestine is limited by antimicrobial factors produced

by Paneth cells, such as defensins and REGIIIg (Moran, Sheehan, and Shanahan 2015; Mowat and Agace 2014). Zoetendal et al (Zoetendal et al. 2012) used healthy ileostomists patients that had their colon removed for over 20 years to study the small intestinal microbiota. They found that there is a decrease in diversity and an increase in carbohydrate metabolizing bacteria in the small intestinal microbiota compared to the colonic microbiota (Zoetendal et al. 2012). Additionally, the small intestinal microbiota fluctuates far more compared to the relatively stable faecal microbiota, most likely in response to dietary variation (Booijink et al. 2010).

Small intestinal bacterial overgrowth

Although the small intestine is not as sterile as has long been believed, an overgrowth of bacteria in the small intestine is still considered pathogenic. The most commonly described definition for small intestinal bacterial overgrowth (SIBO) is a concentration of 10^5 or more colony forming units per millilitre (CFU/mL) of bacteria grown from a small intestinal aspirate (Khoshini et al. 2008). The prevalence of SIBO in the general population is unknown, but is estimated at 0-20% of the otherwise healthy population (Grace et al. 2013). The incidence of SIBO in both intestinal and liver disease has been found to be much higher. For example, great interest has been shown in the role of SIBO in irritable bowel syndrome, with SIBO incidences up to 80% (Aziz, Törnblom, and Simrén 2017). Additionally, a similar link is found between SIBO and non-alcoholic steatohepatitis (NASH) (Ferolla et al. 2014). However, more reliable and robust tests to diagnose SIBO are needed to progress the research in this area (Grace et al. 2013).

1.3 Liver biology

The liver is the largest internal organ and is essential for a large number of metabolic activities, detoxification and nutrient storage (Robinson, Harmon, and O'Farrelly 2016). After this well-regulated absorption of nutrients in the SI, amino acids and carbohydrates are further metabolised in the liver. Where most nutrients are directly transported from the intestine to the liver after absorption, dietary fat bypasses the liver via the lymphatic system. Chylomicrons, produced and secreted by enterocytes into the lymph are exposed to other organs, like the heart and adipose tissue, before chylomicron remnants are taken up by the liver (Whitney and Rolfes 2008). However, most nutrients and other diet-derived components are directly transported to the liver via the portal vein (Welcome 2018). In order to maintain homeostasis, the liver and intestine both need to establish a bidirectional communication. Although rather complex, this communication is believed to primarily occur through signalling molecules produced by these organs and the microbiota living in the intestine. Bile acids play an important role in this enterohepatic communication (see chapter 1.4) (Kuipers, Bloks, and Groen 2014). Dietary chronic overloading of the intestine can disturb this enterohepatic communication, which might be an early cause of the metabolic syndrome and its hepatic manifestation, NAFLD (De Wit et al. 2012).

1.3.1 NAFLD

NAFLD, a co-morbidity of obesity and insulin insensitivity, is the most common hepatic disorder in Western countries and some Asian countries (Fan, Kim, and Wong 2017). At present 20-25% of people in the UK, and over 90% of obese individuals, have NAFLD. NAFLD has emerged as a common risk factor for heart disease, stroke and type two diabetes. If untreated, the milder form of NAFLD will lead to NASH, and in 20% of NASH cases (250,000 current cases) to eventual cirrhosis and liver cancer. Currently, there are no licensed drugs to treat NAFLD/NASH and the only effective treatment includes lifestyle changes (Nascimbeni et al. 2013). Therefore, the identification of an efficacious and affordable strategy for NAFLD management would be of relevance and benefit to a large and ever-increasing proportion of the UK population (British society of Gastroenterology, www.bsg.org.uk).

Dietary interventions have been put forward in the prevention and cure of NAFLD (Roman et al. 2015), for example a carbohydrate-restricted diet has been shown to improve localised fat metabolism in the liver in NAFLD patients (Mardinoglu et al. 2018).

1.4 Bile acids

Bile acids undergo enterohepatic circulation, forming a direct link between the liver and the intestine. Bile acids are natural occurring detergents that play an essential role in the digestion and uptake of dietary fat and fat-soluble vitamins. Bile acids are produced from cholesterol in the liver, conjugated to either taurine or glycine and excreted into the duodenal part of the small intestine. Bile acids have both a hydrophobic and a hydrophilic side which leads to spontaneous micelle formation in aqueous solution. In the lumen of the small intestine, in aqueous solution, Bile acids micelles mix with lipid products resulting from digestion to form mix micelles and subsequently enhance absorption. After most dietary fat is absorbed, around 95% of all bile acids are reabsorbed in the ileum. These bile acids are consequently transported back to the liver via the portal vein, closing the enterohepatic circulation (Schaap, Trauner, and Jansen 2014).

1.4.1 Bile acid metabolism in the liver

In the liver, where bile acids are produced, cholesterol 7 alpha-hydroxylase (CYP7A1) is the rate limiting enzyme in bile acid synthesis. An alternative pathway for bile acid synthesis is via sterol 27-hydroxylase (CYP27A1). Lastly, sterol 12-alpha-hydroxylase (CYP8B1) catalyses the last step in the conversion of cholesterol in cholic acid (CA). Thereby CYP8B1 plays an important role in balance of the two primary bile acids, CA and chenodeoxycholic acid (CDCA). In mice, a shift towards more CDCA leads to an increased production of muricholic acids (MCAs) (Kuipers, Bloks, and Groen 2014). In the small intestine, bile acids can be further metabolised by the gut microbiota, creating secondary bile acids, which is discussed in more detail in chapter 1.4.4. For an overview of the primary and secondary bile acids present in mice and man see table 1.1.

Table 1.1 Overview of bile acids present in the enterohepatic circulation of mice and humans. Colour coding indicates conjugation, white being tauro-conjugated, light grey being glyco-conjugated and dark grey being unconjugated bile acids.

Abbreviation	Name	Primary/ secondary	Mouse/ human
T-a-MCA	Tauro-α-muricholic acid	Primary	Mouse only
T-b-MCA	Tauro-β-muricholic acid	Primary	Mouse only
TCA	Taurocholic acid	Primary	Both (more in mouse)
TCDCA	Taurochenodeoxycholic acid	Primary	Both (more in mouse)
TDCA	taurodeoxycholic acid	Secondary	Both (more in mouse)
TLCA	taurolithocholic acid	Secondary	Both (more in mouse)
GCA	Glycocholic acid	Primary	Both (more in humans)
GCDCA	Glycochenodeoxycholic acid	Primary	Both (more in humans)
GDCA	glycodeoxycholic acid	Secondary	Both (more in humans)
GLCA	glycolithocholic acid	Secondary	Both (more in humans)
a-MCA	α-Muricholic acid	Primary	Mouse only
b-MCA	β-Muricholic acid	Primary	Mouse only
CA	Cholic acid	Primary	Both
CDCA	Chenodeoxycholic acid	Primary	Both
DCA	deoxycholic acid	Secondary	Both
UDCA	Ursodeoxycholic acid	Secondary	Both
LCA	Lithocholic acid	Secondary	Both
ϖ-MCA	ϖ-Muricholic acid	Secondary	Mouse only

In addition to the differences in bile acid pools between mice and humans, consisting of mostly hydrophobic bile acids in mice and more hydrophobic bile acids in humans, regulation of bile acid synthesis from the precursor cholesterol also differs (Chiang 2009). In rodents, the proximal promotor of the CYP7A1 gene binds the oxysterol activated liver X receptor (LXRα) while this binding does not exist in humans (Janowski et al. 1996). This binding leads to an increase in bile acid synthesis in mice on a high cholesterol diet, which could not be observed in LXR knockout mice (Peet et al. 1998). Further confirmation was found in transgenic mice carrying the human CYP7A1 gene, which did not show an increase in bile acid excretion in response to a high cholesterol diet (Chen et al. 2002). Taken together, this indicates different regulation of bile acids synthesis between mice and humans and a stronger influence of the cholesterol content of the diet on bile acid metabolism in mice. It is important to keep this in mind during the interpretation of bile acid differences observed in mouse studies.

1.4.2 Bile acid metabolism in the ileum

Reabsorption of bile acids in the ileum is largely the consequence of active uptake of bile acids via the apical sodium dependent bile acid transporter (ASBT). In ileal enterocytes, bile acids are sensed by the transcription factor farnesoid X receptor (FXR). FXR regulates the expression of genes that play a role in intracellular transport of bile acids (fatty acid binding protein 6 (FABP6)) and genes that regulate the transport of these bile acids into the bloodstream (Organic solute transporter alpha/ beta (OSTα/β)) (Dawson and Karpen 2015; Thomas et al. 2010). Furthermore, in 2005 it has been shown that FXR induces the expression of the signalling molecule fibroblast growth factor 19 (FGF19, with *Fgf15* being the homologue in mice) (Inagaki et al. 2005). FGF15/19 travels in the bloodstream to the liver where it binds to fibroblast growth factor receptor 4 (FGFR4) and via a cascade of reactions blocks the expression of CYP7A1, the rate limiting enzyme in *de novo* bile acid synthesis (Inagaki et al. 2005; T. Li and Chiang 2015). Additionally, re-absorbed bile salts that reach the liver activate hepatic FXR and block CYP7A1 via small heterodimer partner (SHP). For a basic overview of enterohepatic bile acid metabolism, please see figure 1.1.

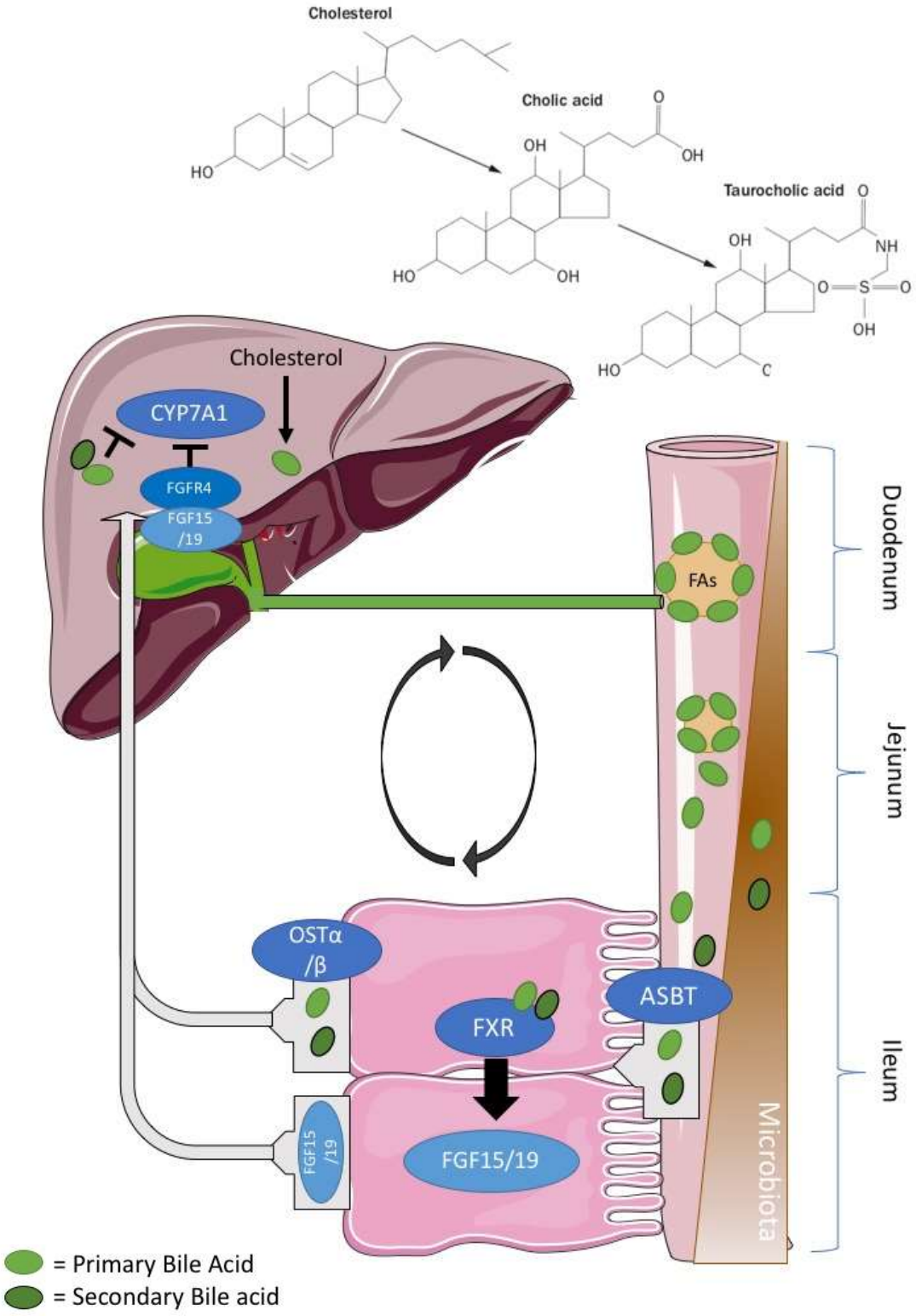
Cholesterol
Cholic acid
Taurocholic acid
Cholesterol
CYP7A1
FGFR4
FGF15
/19
FAs
Duodenum
Jejunum
Ileum
OSTα
/β
FXR
ASBT
FGF15/19
FGF15
/19
Microbiota
= Primary Bile Acid
= Secondary Bile acid

Figure 1.1 Simplistic overview of bile acid metabolism in the liver and small intestine. Upper figure (chemical structures) is adapted from (Kuipers, Bloks, and Groen 2014). Bile acids are produced from cholesterol in the liver and excreted into the duodenal part of the small intestine. Here they play an essential role in the uptake of dietary fat and fat-soluble vitamins. In the ileum, bile acids are reabsorbed and activate the bile acid sensing transcription factor Farnesoid X Receptor (FXR). FXR induces the expression of fibroblast growth factor 15/19 (FGF15/19) which travels to the liver via the portal vein and inhibits the rate limiting enzyme in de novo bile acid synthesis: Cholesterol 7 alpha-hydroxylase (CYP7A1). (FA= fatty acid, ASBT= apical sodium dependent bile acid transporter, OSTα/β= Organic solute transporter alpha/beta)

Synthetic activation of FXR by GW4064 gives opposing results depending on its route of administration. If GW4064 is given via an IP injection, the activation of FXR is protective against diet induced obesity (DIO) and the development of NAFLD (Ma et al. 2013; Zhang et al. 2006). However, if GW4064 is given orally it increases DIO and hepatic triglycerides (TG) accumulation (Watanabe et al. 2011). This indicates that intestinal FXR activation provokes differential effects in comparison with hepatic FXR activation. In line with this, intestinal specific FXR knockout mice are protected against NAFLD on a 1% cholesterol diet in contrast to hepatic FXR knockout mice (Schmitt et al. 2014). Jiang *et al* even found that intestinal specific FXR knockout mice are less prone to develop NAFLD on a HF diet, most likely due to a reduction in circulating ceramides (Jiang et al. 2015). Contrastingly, intestine-specific FXR agonist fexaramine reduces DIO and insulin resistance while promoting browning of white adipose tissue. Intestinal FXR activation leads to a large increase in FGF19/15 expression and thereby alters the bile acids composition, possibly in a way that might explain these positive findings (Fang et al. 2015). In conclusion, although molecular mechanisms are partly unknown, FXR signalling in both the small intestine and the liver has strong implications for enterohepatic health.

The importance of bile salt and FXR metabolism has recently been emphasized in bariatric surgery research (Penney et al. 2015). In bariatric surgery, positive metabolic changes occur even before weight loss. It is also known that bariatric surgery increases circulating bile salts (Myronovych et al. 2014). Recently, a study by Ryan *et al* (Ryan et al. 2014) showed that the beneficial effects of vertical sleeve gastrectomy (VSG) surgery are FXR dependent. FXR knockout mice regain weight within 8 weeks after the surgery and lose glycaemic improvement. Unfortunately, whole body FXR knockout mice were used in

this study, so it is impossible to distinguish between intestinal and hepatic FXR (Ryan et al. 2014).

1.4.3 Bile acid metabolism in the rest of the body

Besides activating FXR, bile salts are also capable of activating TGR5, a G-protein coupled receptor that is expressed throughout the body (Yuanyuan Li, Jadhav, and Zhang 2013). In mice, bile acids activate TGR5 in brown adipose tissue leading to increased energy expenditure (Watanabe et al. 2006). In humans, TGR5 expression in subcutaneous adipose tissue correlates with resting metabolic rate (Svensson et al. 2013). Recently, Ullmer *et al* found that TGR5 activation leads to an increase in the gut derived hormones PYY and GLP-1 and exerts positive effects on glucose tolerance (Ullmer et al. 2013). This indicated that changes in bile acids and bile acid metabolism could provoke health effects beyond enterohepatic health.

1.4.4 Bile acids and the microbiota

There is a two-way interaction between the gut microbiota and bile acids. First, bile acids exert antimicrobial properties, with some bacterial species being more prone to these effects than other. Bile acid feeding increases the amount of *Firmicutes* while decreasing the amount of *Bacteroides*, a similar pattern as is observed after the consumption of a high fat diet (Murphy, Velazquez, and Herbert 2015). Second, bacteria are capable of deconjugating and altering the structure of bile acids, forming secondary bile acids (Ridlon et al. 2014). The enzyme bile salt hydrolase (BSH) is responsible for the conversion of primary bile salts (as produced by the liver) into secondary. BSH is a unique enzyme of the gut microbiota and is distributed across the major bacterial species in the gut (Jones et al. 2008). While most bile salts are FXR agonists, the primary bile salt Tauro-beta-muricholic acid (T-β-MCA) is an FXR antagonist. Due to the lack of intestinal bacteria, no BSH is present in germ free mice. Consequently, T-β-MCA cannot be deconjugated and reaches the ileum where it blocks FXR. This results in reduced expression of *Fgf15* and an increase in bile acid synthesis in the liver (Sayin et al. 2013a). Administrating BSH to germ free mice counteracts these effects (Joyce et al. 2014). In humans, it was shown that 1 week of Vancomycin treatment decreases gram positive bacteria like Firmicutes.

These changes lead to a reduced conversion of primary bile salts into secondary bile salt and decreases circulating FGF19 (Vrieze et al. 2014). Besides antibiotics, nutritional compounds are also capable of changing the microbiota. Li et al showed that the antioxidant tempol alters the microbiota in a way that more T-β-MCA is present in the ileum (Li et al. 2013). Mice on tempol have decreased intestinal FXR expression and are protected against DIO. This is in consonance with the finding that intestine-specific FXR knockout mice are protected against NAFLD (Jiang et al. 2015). Conversely, conventionally raised mice administered with BSH show a dramatic reduction in T-β-MCA and are also protected against DIO (Joyce et al. 2014). For an overview of the primary and secondary bile acids see table 1.

The two-way interaction between the microbiota and bile acids is not only important in enterohepatic communication, but also in the rest of the body, as the gut microbiota modulation determines bile acid profiles throughout the body (heart, kidney). Here they can have signalling properties and influence cardiometabolic health (Swann et al. 2011). Additionally, microbiota changes by bile acids can also influence cardiometabolic health. Both via the altered production of SCFAs and by the production of atherosclerotic promoting molecules like trimethylamine (TMA) (Janssen and Kersten 2015). Increased serum concentrations of the liver metabolite of TMA, TMAO, are linked to atherosclerosis (Gregory et al. 2015). Furthermore, NAFLD and liver cirrhosis are associated with an altered microbiota. A recent study has found an increased presence of buccal derived bacteria in the gut in liver cirrhosis patients (Qin et al. 2014). It is known that bile acids are altered in NAFLD patients (Lake et al. 2013), which might be the cause of the alterations is microbiota composition.

1.4.5 Bile acids and diets

Bile acids play an essential role in the uptake of dietary fat, however, the effect of a high fat diet on the bile acid pool remains partly unclear. It is hypothesized that a HF diet increases the bile acid pool because of the crucial role of bile acids in lipid digestion. However, Ostα-/- mice have half the bile acid pool of wild type animals but only lose minimum amount of lipids in the faeces on a HF diet (Hammond et al. 2015). This may indicate that an excess of bile salts is present in wild type animals. In both humans and

mice, a HF diet increases faecal bile acids (Stenman, Holma, and Korpela 2012; Bianchini et al. 1989), but this is the small percentage of bile acids that is not reabsorbed in the ileum. Furthermore, these bile acids have travelled through the colon where microbiota have altered their structure. Arguably, finding out what happens to bile acid metabolism in the small intestine in response to a high fat diet might be more relevant.

Another dietary component linked to bile acid metabolism is dietary fibre. Dietary fibres are known for their cholesterol-lowering effects, and have been hypothesized to bind bile acids and avoid their absorption leading to more conversion of cholesterol into bile acids (more in chapter 1.4.3).

Additionally, the composition of the microbiota can be influenced by alterations in the diet. Switching between animal and plant-derived food shows large alterations in the microbiota and microbiota-derived signalling molecules. These effects can already be seen after 5 days in humans (David et al. 2014) and after 3.5 days in mice (Carmody et al. 2014). In mice, diet is even found to be a more important determinant of the composition of the microbiome than genotype (Carmody et al. 2014). Since the microbiota plays a very important role in bile acid metabolism, it is not hard to speculate that this will also influence bile acids. Dietary fibres are known to alter the composition of the microbiota leading an increased production of short chain fatty acids (Lange et al. 2015).

In both human and mice studies, a HF or animal-derived diet increases the presence of bile tolerant bacteria (mainly Firmicutes), while decreasing bacterial populations that produce SCFA (mainly Bacteroides) (David et al. 2014; O'Keefe et al. 2015; Turnbaugh et al. 2008; Hildebrandt et al. 2009). Only saturated fat seems to provoke these microbial changes, probably due to less efficient uptake and overflow of saturated fatty acids into the distal part of the intestine (N. de Wit et al. 2012; Patterson et al. 2014). Interestingly, bile acid feeding provokes similar changes in microbiota as a HF diet (Islam et al. 2011), supporting the theory that diet affects bile acid metabolism.

1.5 FGF15/19

In humans, seven functional subfamilies of fibroblast growth factors (FGFs) have been identified (Beenken and Mohammadi 2009). Classically, FGFs are intracrine or paracrine factors, however the FGF19 subfamily (consisting of FGF19, FGF21 and FGF23) is an atypical group of FGFs that lack a heparin binding domain. This allows them to be secreted into the bloodstream. Therefore, FGF19 and FGF21 are endocrine factors that are often considered as metabolic hormones. All paracrine and endocrine FGFs bind to their receptors to exert their function. Four of these FGF receptors (FGFRs), namely FGFR1 to FGFR4, have been identified. FGF19 potently binds to FGFR4, but has also been shown to have affinity with FGFR1. Additionally, FGF19 needs a membrane-bound cofactor β-Klotho to successfully bind to and activate FGFRs (Markan and Potthoff 2016; Kliewer and Mangelsdorf 2015).

The mouse homologue of FGF19 is called *Fgf15*. This difference in terminology is due to the lack of common amino acid identity; at the time of cloning it was found that the proteins only share 53% of amino acids (Nishimura et al. 1999). However, functional studies have shown that *Fgf15* and FGF19 are orthologous proteins with a conserved FXR binding site in the promoter regions of the encoding genes (Kliewer and Mangelsdorf 2015). For that reason, we will be referring to the FGF15/19 protein throughout this thesis.

The highest expression of FGF15/19 is in the ileum, where bile acids are reabsorbed. Here, FGF15/19 is exclusively expressed in enterocytes (Haber et al. 2017). FGF15/19 secretion is often considered a postprandial response, with a peak of circulating FGF19 levels in humans found approximately 60 mins after consumption of a meal (Potthoff et al. 2011). Postprandially, FGF15/19 regulates bile acid, carbohydrate and protein metabolism. Although FGF15/19 is mostly known to inhibit bile acid synthesis by blocking hepatic CYP7A1 expression, FGF15/19 has also been shown to activate both protein and glycogen synthesis in the liver (Kir et al. 2011). FGF15/19 is capable of lowering blood glucose levels independent of insulin (Wu et al. 2010), via inhibiting the CREB-PGC-1a pathway in the liver (Potthoff et al. 2011).

1.5.1 FGF15/19 in health and disease

Due to its nature as an metabolic hormone, many health effects have been linked to the over and under expression of FGF15/19 (Jahn et al. 2015). For example, a mouse model designed to express FGF19 via a myosin light chain promotor, expressing FGF19 mostly in muscle tissue, showed a decrease in body weight on both a chow and a HF diet. Additionally, lower fat mass, more brown adipose tissue and less triglyceride storage in the liver was observed (Tomlinson et al. 2011). Furthermore, mice fed with a HFD and injected with FGF19 had reduced body weight, were protected against diabetes and showed also decreased liver triglycerides (Fu et al. 2004). Additionally, *Fgf15* knockout mice are high responders to high fat diets, gaining significantly more weight than their wild type litter mates while accumulating more triglycerides in the liver (Alvarez-Sola et al. 2017). *Fgfr4* knockout mice, lacking the receptor for FGF15/19 signalling, develop signs of the metabolic syndrome while on a chow diet, including increased adipose tissue, glucose intolerance and hyperlipidaemia (Huang et al. 2007). In humans with NAFLD, type 2 diabetes and the metabolic syndrome FGF19 levels in serum are shown to be lower than in healthy controls (Barutcuoglu et al. 2011; Wojcik et al. 2012; Gallego-Escuredo et al. 2015; Eren et al. 2012).

NAFLD

Due to the response of both FGF19 overexpressing and *Fgf15* KO mice to HF diets, interest has developed in the preventive and restorative role of FGF15/19 in NAFLD. Intestinal specific FXR activation via the FXR agonist fexaramine, which strongly induces the expression of *Fgf15*, reduces insulin resistance while promoting browning of white adipose tissue in mice (Fang et al. 2015). Additionally, *Fgf15* has been shown to repress the expression of Pparg2 in the liver, a transcription factor contributing to high fat induced hepatic steatosis (Alvarez-Sola et al. 2017). The biggest struggle in the development of FGF15/19 as a drug in NAFLD has been the carcinogenic properties of FGF15/19, as will be discussed in the next section. Recently, a non-carcinogenic engineered FGF19 analogue, NMG282, has been developed (M. Zhou et al. 2014). NGM282 seems a very promising treatment for NAFLD as it has been shown to be effective at reversing NAFLD

in humans by reducing both liver fat content and markers of liver inflammation (Harrison et al. 2018).

Hepatic carcinoma

FGF15/19 binding to hepatic FGFR4 leads to an increase in cell proliferation in the liver (Repana and Ross 2015). This can be hepatoprotective during active liver injury, as has been shown after partial hepatectomy in mice (Uriarte et al. 2013). However, when FGF15/19 is chronically activated it is considered pro-carcinogenic (Repana and Ross 2015). FGF19 expression in human hepatic tumour cells correlates with poor progression of liver cancer phenotypes (Miura et al. 2012; Hyeon et al. 2013; Hagel et al. 2015) and *Fgf15* KO mice develop less hepatic carcinoma under treatment of diethyl nitrosamine. Furthermore, *Fgf15* KO mice show less cell proliferation in the liver, as shown by Ki-67 and AFP and less fibrosis as shown by SMA (Uriarte et al. 2015). Interfering with the FGF19 – FGFR4/KLB signalling pathway in liver cancer is regarded a new strategy in fighting this disease (Alvarez-Sola et al. 2017).

Bile acid diarrhoea

Bile acid diarrhoea (BAD) is described as chronic diarrhoea in response to excessive loss of bile acid in faeces (Walters 2014). BAD is often wrongly diagnosed as diarrhoea-predominant irritable bowel syndrome (IBS-D) (Spiller and Thompson 2010), with around 30% of IBS-D patients showing abnormal faecal bile acid loss (Wedlake et al. 2009).
The role for enterohepatic communication via FGF15/19 has been emerging in BAD. In FGF15 KO mice an increase in faecal bile acid excretion was found (Inagaki et al. 2005), while monkeys treated with anti-FGF19 antibodies developed severe diarrhoea (Pai et al. 2012). In humans, bile acid absorption seems to be unaffected in most BAD patients, and an increased bile acid pool has been shown. However, circulating FGF19 levels are decreased in BAD patients (Walters et al. 2009) and possible intervening by FGF19 induction is considered a promising therapeutic approach for BAD patients (Walters 2014). Interestingly, the previously-discussed non-carcinogenic FGF19 analogue NGM282 has recently been shown to increase gastro-intestinal transit time (Oduyebo et al. 2018).

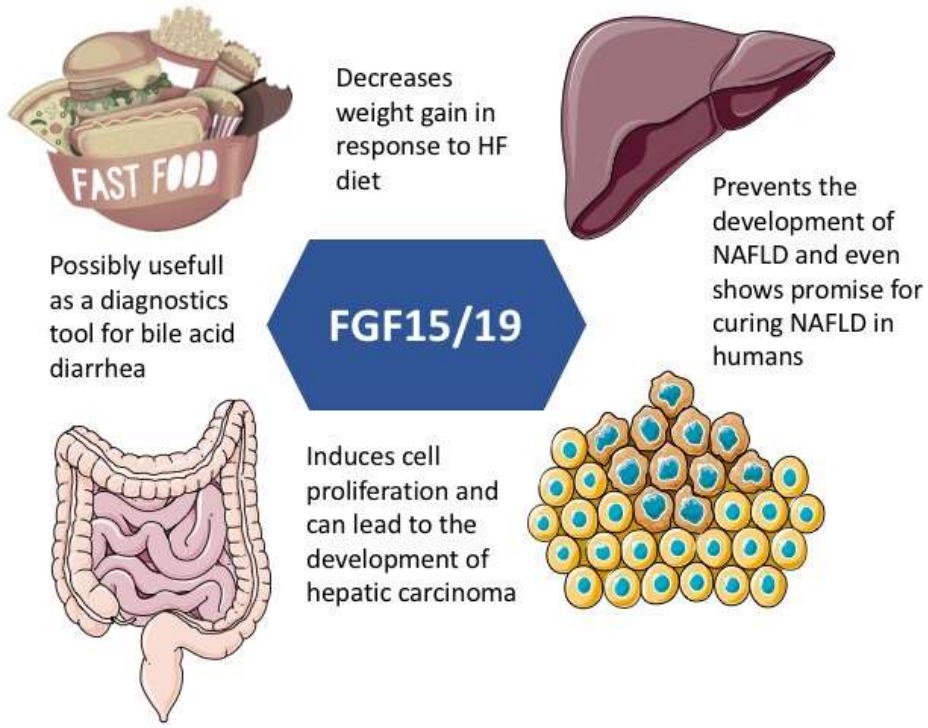

Figure 1.2 Summary of health effects of FGF15/19 throughout the body.

1.5.2 Regulation of FGF15/19

As discussed previously, classically, FGF15/19 is regulated by the bile acid sensing transcription factor FXR and activity of FXR can be modulated by conversions of bile acids by the microbiota (Kuipers, Bloks, and Groen 2014). In this section, alternative regulation of FGF15/19 will be discussed.

Firstly, the fat-soluble vitamins A and D have been linked to an increase in FGF15/19 expression. Vitamin D KO mice show a decrease in *Fgf15* expression, while wild type mice fed with vitamin D show increased expression of *Fgf15*. This effect was independent of FXR as shown by *Fxr* KO mice. Additionally, vitamin A feeding also leads to an increased expression of *Fgf15*, however, this effect was obliterated in FXR KO mice (Schmidt et al. 2010).

Furthermore, the transcription factor PPARα influences FGF15/19 expression. PPARα in the small intestine regulates the expression of UDP-glucurosyltransferase (UGT) genes (Ugt1a1, Ugt1a7, Ugt2b35) which are proteins capable of glucuronidation of bile acids. This, when reaching the ileum, leads to lowered FXR activation and a corresponding decrease in *Fgf15* expression. Additionally, *Ppara* KO mice have increased ileal *Fgf15* expression and decreased hepatic Cyp7a1 expression (Zhou et al. 2014). The PPARA agonist clofibrate has been shown to increase bile acid excretion, decrease *Fgf15* expression and increase hepatic bile acid production via Cyp7a1 (Youcai Zhang et al. 2017).

Diet1 is a newly identified gene in the ileum that induces the expression of *Fgf15*. Mice with a mutation in the Diet1 gene have less *Fgf15* expression and as a consequence higher *Cyp7a1* expression and bile acid pools. DIET1 and FGF15 proteins are in close proximity from each other within the cell, suggesting a direct interaction (Vergnes et al. 2013).

Lastly, bile acid production via *Cyp7a1* is regulated by circadian rhythms and is highest in mice at the start of the dark phase. Circadian regulation is driven by the transcription factor Kruppel-like factor 15 (*Klf15*) in the ileum where it downregulates the expression of FGF15/19. The expression of *Klf15* follows a pattern over 24h that is opposite to the expression pattern of *Fgf15* and in *Klf15* KO mice expression of *Fgf15* is upregulated (Han et al. 2015).

1.6 Digestible carbohydrates: sugar and starch digestion

Although the knowledge of the effect of diet on bile acid metabolism is limited (see chapter 1.4.5), the interaction between carbohydrates and bile acid metabolism is interesting. On the one hand *Fgf15* has been shown to be able to alter glucose metabolism (Potthoff et al. 2011), while on the other hand the effects of mostly undigestible carbohydrates on the gut microbiota might alter bile acid metabolism (Li and Chiang 2015). Because these effects will be studied further in this thesis, this and the following sub-chapter will give some background on digestible and undigestible carbohydrates and their effects on the gut and liver.

1.6.1 The role of carbohydrates in human nutrition

Carbohydrates generally form the biggest food group in the standard western diet, accounting for around 43% of total calories in European countries (Vergnaud et al. 2013). Digestible carbohydrates can be divided into simple carbohydrates (the sugars) and complex carbohydrates (starches). Both digestible and indigestible carbohydrates are polymers built up from monosaccharide units. Three monosaccharides important for human nutrition are glucose, fructose and galactose which all possess the same chemical formula $C_6H_{12}O_6$. Structurally, the monosaccharides differ in taste perception with fructose being the sweetest. In nutrition, sugars are usually in disaccharide form, with the most important disaccharides being sucrose (glucose and fructose), maltose (glucose and glucose) and lactose (glucose and galactose). The most important form of complex digestible carbohydrate is starch, a long, branched or unbranched chain of hundreds or thousands of glucose molecules linked together. Starches can be found in plant-based foods, as they are a way of storing glucose. Animals store glucose as glycogen, a highly branched chain of glucose molecules, in the liver and muscle tissue. However, glycogen in animal muscle rapidly hydrolyses after slaughter, making it an insignificant dietary source of carbohydrates (Whitney and Rolfes 2008; Welcome 2018).

1.6.2 Digestion of carbohydrates

In order to be able to absorb dietary carbohydrates they are broken up into monosaccharides in the GI tract. Mono- and disaccharide digestion is a fast process and

occurs mostly in the duodenum. Monosaccharides can be absorbed directly, while disaccharides and small oligosaccharides are cleaved into monosaccharides by enzyme complexes called α-glucosidases (Rosak and Mertes 2012). α-Glucosidases are membrane bound enzymes present in the epithelium throughout the small intestine. The most important α-glucosidases present in the human small intestine are lactase, maltase-glucoamylase (MGAM) and sucrase-isomaltase (SIS). Lactase is responsible for the breakdown of the disaccharide lactose, while MGAM breaks down maltose and glucose oligosaccharides, lastly SIS breaks down sucrose and glucose polymers (Tundis, Loizzo, and Menichini 2010; Quezada-Calvillo et al. 2008).

More complex carbohydrate digestion starts in the mouth, where salivary amylase cleaves a small percentage of the present starches into smaller polysaccharides and the disaccharide maltose. Further cleavage of starch happens in the upper part of the small intestine, where pancreatic amylase is added to the food bolus. Amylase catalyses the hydrolysis of α-1,4-glucan bonds present in starch and maltodextrins (Svensson 1988). When starch has broken into short enough chains of glucose, α-glucosidases on the outer membrane of the intestinal cells break it down into monosaccharides which are consequently absorbed. A high starch (HS) diet has been shown to increase the activity of α-glucosidases in the more downstream parts of the small intestine (Goda 2000).

1.6.3 Glucose and fructose metabolism in the small intestine

After starch and sucrose digestion, glucose is absorbed into the intestinal epithelial cells by the sodium-glucose linked transporter (SGLT1) which is localized in the apical brush border membrane (Gorboulev et al. 2012). Another glucose transporter, glucose transporter 2 (GLUT2), primarily mediates the lateral transport of glucose into the bloodstream, but deletion of GLUT2 has been shown to also decrease apical glucose absorption (Schmitt et al. 2017). After absorption into the enterocytes glucose is excreted into the portal vein by GLUT2. Although the glucose transporters SGLT1 and GLUT are not under direct control of insulin, intestinal deletion of the insulin receptor does decrease glucose absorption (Ussar et al. 2017), indicating a role for insulin. Additionally, under the control of insulin, the small intestine is capable of endogenous production of glucose,

making it a gluconeogenic organ like the liver and kidneys (Mithieux and Gautier-Stein 2014; Croset et al. 2001).

Fructose is absorbed into the intestinal epithelial cells via the transporter GLUT5 (Douard and Ferraris 2008). Although it was long believed that fructose is consequently transported to the liver, recently it has been shown that the small intestine converts around 90% of all fructose into glucose, galactose and glycerate if a low to medium dose of fructose is consumed (Jang et al. 2018).

1.6.4 Glucose metabolism in the liver

When glucose reaches the liver via the portal vein it is taken up into the hepatocytes via GLUT2 (Takeda et al. 1993). Consequently, all glucose is phosphorylated to glucose 6-phosphate by glucokinase after which several metabolic pathways can be initiated. Postprandially, most glucose 6-phosphate is converted into glycogen. Alternatively, a surplus of glucose might be used to synthesize *de novo* fatty acids via acetyl-CoA conversion. Lastly, glucose can be used produce NADPH and 5-phosphate via the pentose phosphate pathway (Adeva-Andany et al. 2016).
During short-term periods of fasting, the liver mainly uses its glycogen storages to provide other organs of glucose. During longer periods of fasting, the liver produces *do novo* glucose via gluconeogenesis (Rui 2014).

1.7 Undigestible carbohydrates: dietary fibres

Dietary fibres (called fibres from hereon) are remnants of plant cells that cannot be degraded by enzymes produced by humans (Trowell 1976) and are also defined as undigestible carbohydrates. In the UK, the recommended daily intake is 30 grams, however the average consumption is only around 20 grams per day (Spencer et al. 2003). Fibres can be found in plant-based foods only and in the Western world total fibre consumption comes 50% from grains, 30-40% from vegetables, 16% from fruit and the remaining from minor sources (Dhingra et al. 2012).

1.7.1 Soluble versus insoluble fibres

Fibres can be roughly classified into insoluble and soluble fibre. Insoluble fibre, as the name suggests, cannot be dissolved in water. They mostly resist degradation by the microbiota and are therefore labelled less fermentable. Soluble fibres dissolve in water and form gel-like structures delaying nutrient absorption. Soluble fibres are fermented by the gut microbiota which leads to the production of short chain fatty acids (SCFAs) (Aleixandre and Miguel 2008; Cockburn and Koropatkin 2016). For an overview of insoluble and soluble fibres and their gradation of fermentability, see table 1.2.

Table 1.2 Overview of selected dietary fibres. This table is based on the publication by Aleixandre *et al* (Aleixandre and Miguel 2008).

Fibre	Insoluble/soluble	Fermentability
Cellulose	Insoluble	Poor
Hemicellulose	Insoluble	Intermediate
Lignin	Insoluble	Poor
Pectin	Soluble	Intermediate
Guar gum	Soluble	High
Inulin	Soluble	High
Fructo-oligo saccharides (FOS)	Soluble	High
B-glucans	Soluble	High

1.7.2 Fibres and the microbiota

Fibre consumption provokes changes in the gut microbiota and some fibres are classified as prebiotics. Prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al. 2017). Under this definition, almost all prebiotics are fibres, however not all fibres are prebiotics. A lack of dietary fibres can decrease the microbial diversity in the gut (Sonnenburg et al. 2016), something that also happens when in response to modernisation of hunter-gathers communities (Smits et al. 2017). Lange *et al* showed that four fermentable fibres (inulin, FOS, arabinoxylan and guar gum) provoke similar changes to the microbiota, which are different from mice fed less fermentable resistant starch. Host colonic gene expression was also similar in response to all fermentable fibres, caused by an activation of Pparg by the SCFAs produced (Lange et al. 2015). Over generations, the lack of dietary fibres, as can be found in a western style diet, can irreversibly alter the composition of the gut microbiota (Sonnenburg et al. 2016).

Although to a lesser extent, SCFAs are also produced from soluble fibres by the microbiota of the small intestine. SCFAs are essential for the functionality of the SI as is shown by the importance of SCFA for proper vitamin A uptake (Goverse et al. 2017). A lack of dietary fibre and therefor of SCFA could potentially lead to malabsorption of vitamin A.

1.7.3 Health effects of dietary fibres

The health benefits of dietary fibres are indisputable. Fibre consumption has been shown to decrease all-cause mortality (Yang et al. 2015), protects against colon cancer (Ben et al. 2014) and decreases the incidence of cardiovascular disease and coronary heart disease (Threapleton et al. 2013). This is most likely due to the cholesterol lowering effects of soluble fibres, with oat products, psyllium, pectin and guar gum all linked to a decrease in both total and LDL cholesterol levels (Brown et al. 1999). The cholesterol-lowering effects of β-glucans in oats were confirmed more recently in humans (Whitehead et al. 2014) and pigs (Gunness et al. 2016).

Most research addressing the molecular effects of fibres have been focused on the colon, showing alterations in the microbiota and SCFA production, colonic gene expression

(Lange et al. 2015), alterations in the colonic mucus layer (Schroeder et al. 2018) and the induction of IL-22 in colonic enterocytes (Zou et al. 2018). However, their role in the small intestine is less elucidated.

1.8 Healthy ageing

Some of the most debilitating diseases, like cardiovascular disease, cancer and neurodegenerative diseases share age as a main risk factor (Niccoli and Partridge 2012). With healthy ageing arguably being a goal for all individuals, there is a lot of scientific interest in slowing the ageing process. This would delay both the onset and the progression of all age related diseases (Kaeberlein, Rabinovitch, and Martin 2015). The ageing process, here defined as the minimization of physical impairments, has an estimated genetic prediction of 30 to 60% with the remaining being determined by environmental factors (Foebel and Pedersen 2016). Since human environments are complex and potentially highly modifiable, much research focuses on influencing key environmental factors, one of which is nutrition.

1.8.1 Expanding life span

From the currently-available methods, calorie restriction (CR) is the most studied intervention for delaying the ageing process. CR is defined as a reduction in energy intake while preventing malnutrition and has been shown to increase the life-span of a wide range of species, from yeast to mice and primates (Fontana, Partridge, and Longo 2010; Fontana and Partridge 2015). Most likely due to the difficulty of studying long term CR in humans, the results in voluntary subjects are inconsistent. Cardiovascular health seems to benefit from long term CR in humans (Stein et al. 2012), while the delays in immune systems ageing cannot be replicated in humans (Tomiyama et al. 2017).

Although CR shows some promising effects in humans, the dietary regime is demanding and difficult to maintain. However, the beneficial effects observed from CR has spurred research to search for alternatives, mainly in the form of small molecule CR mimetics. For example, some widely used anti-diabetes drugs, namely metformin and acarbose, have been put forwards as CR mimetics. These drugs both alter glucose metabolism, metformin in the liver and acarbose in the intestine, and thereby mimic CR. Metformin has been shown to increase healthy ageing in mice, especially in combination with other bioactive components (De Haes et al. 2014; Strong et al. 2016) and diabetic patients receiving metformin have reduced mortality (Bannister et al. 2014). Metformin is currently being tested in a large multicentre clinical trial with 3000 elderly subject to further investigate

its effects on ageing (Barzilai et al. 2016). Acarbose has been shown to increase life-spam in mice, with a large effect in male mice and modest effect in females (Harrison et al. 2014). Other promising small molecules for healthy ageing include the mTOR inhibitor rapamycin (Ehninger, Neff, and Xie 2014) and sirtuin activators (Mitchell et al. 2014).

1.8.2 Bile acids and ageing

Ageing affects almost all metabolic systems in the body, so also bile acid metabolism. For example, during ageing the conversion of cholesterol to bile acids in the liver decreases (Einarsson et al. 1985). Which is most likely due to a lower activity of cholesterol 7 alpha-hydroxylation, the rate limiting step in this conversion (Bertolotti et al. 1993). Additionally, bile acid excretion into the faeces decreases in ageing rats, which could be partially reversed by a 40% CR diet (Ferland et al. 1989). Conversely, an increase in tauro-conjugated bile acid were found in the gallbladders of aged rats compared to their younger counterparts (Lee et al. 2016).

Bile acids have also been studied for their influence on the ageing process (Krøll 2012). The conjugated secondary bile acid tauro-ursodeoxycholic acid (TUDCA) has been shown to have a number of beneficial health effects. For example, TUDCA normalizes the glucose intolerance associated with a HF diet (da-Silva et al. 2011). Additionally, during partial hepatectomy, TUDCA improves liver regeneration and reduces inflammation, apoptosis and necrosis (Mosbah et al. 2010). Additionally, unconjugated UDCA is often used in the treatment of cholestatic liver disease, a condition where bile acids accumulate in the hepatocytes (Paumgartner and Beuers 2002). Furthermore, TUDCA has been shown to alleviate myocardial contractile dysfunction in obese mice (Ceylan-Isik, Sreejayan, and Ren 2011). Taken together, both conjugated and unconjugated UDCA are capable of reducing the incidence of a number of age-related and metabolic diseases.

Lastly, there are indications that specific bile acids can prolong life-span (Roux and Chartrand 2010). In yeast, the bile acid lithocholic acid (LCA) have been shown to increase life-span via an mTOR dependent pathway (Goldberg et al. 2010). Furthermore, the bile-acid like compound dafachronic acid has been shown to increase life span in C.

elegans via activation of the nuclear receptor DAF-12, which is similar to FXR in vertebrates (Gerisch et al. 2007). Later it has been shown that the C. *elegans* homologue of LXR (NHR-8) for this mechanism (Magner et al. 2013; Groen and Kuipers 2013). Ultimately, it has been hypothesized that the increased level of bile acids in the long-lived *Ghrhr* mutant mouse models contribute to its increased longevity (Gems 2007).

1.9 Mouse models in biomedical research

Although research in humans is the preferred option, it is difficult to investigate causality in humans due to the difficulty of obtaining samples. As an alternative, murine models are heavily used in biomedical research. Mice provide a good, tractable animal experimental model of human intestinal biology with many of the key physiological features of the human GI-tract including the architecture, organisation and functions of the intestinal epithelium, microbiota and gut-associated lymphoid tissue faithfully reproduced in mice. Additionally, mice genetics have been widely studied and genetically modified mice are available for functional studies. Moreover, studies are relatively cheap due to the low maintenance cost and short life cycle of mice (Nguyen et al. 2015).

1.9.1 Diets used in mouse studies

While mice used in biomedical research are inbred and therefore genetically identical, reproducibility between murine studies is not optimal. This is at least in part due to the lack of good control diets. A common and arguably most employed chow diet is produced from unrefined plant products, which leads to large variations between vendors, seasons and even batches. The current alternative to the chow diet is a purified control diet, which is stable and defined in composition (Pellizzon and Ricci 2018). Unfortunately, the effects of the purified diet on the health of mice haven't been properly investigated. The purified diet has been shown to lack in aryl hydrocarbon receptor (AHR) ligands, which is detrimental for SI health (Ying Li et al. 2011). Additionally, the purified diet does not contain any soluble fibre only cellulose, possibly leading to changes in the microbiota and mouse health. Although proper control diets are essential in any field of research, molecular nutrition research and research investigating the role of the intestinal microbiota might be extra sensitive.

1.10 Identifying research gaps

As discussed previously, little is known about the effects of dietary patterns on bile acid metabolism. The indications that carbohydrates might predominantly influence bile acid metabolism are plentiful. First, it is well established that bile acid metabolisms, via the signalling molecule FGF15/19 influences carbohydrate metabolism in the liver (Kir et al. 2011; Potthoff et al. 2011). Secondly, FGF15/19 is a postprandial signal (Potthoff et al. 2011) indicating an opportunity for dietary regulation. Additionally, it is known that dietary components can alter the microbiota is such a way that it changes ileal bile acid metabolism (F. Li et al. 2013) and undigestible carbohydrates are known to severely influence the composition of the microbiota (Makki et al. 2018). Lastly, undigestible carbohydrates lower blood cholesterol levels, which has been previously been hypothesised to be due to alterations in bile acid metabolism (Gunness et al. 2016). Because of these indications that dietary patterns, and especially digestible and undigestible carbohydrates, could potentially influence bile acid metabolism, this is further investigated in this thesis.

In order to investigate this, the aim of this thesis have been defined to:

1. Investigate the effects of digestible carbohydrates on bile acid metabolism in the small intestine
2. Investigate the effects of undigestible carbohydrates on bile acid metabolism in the small intestine
3. Investigate the effects of digestible versus undigestible carbohydrates on the gut-liver axis
4. Investigate bile acid metabolism during healthy ageing, using CR as a model

To study effects of diets on bile acid metabolism we first aim to study the role of digestible carbohydrates on bile acids metabolism in chapter 3. Here we compare the standard chow diet to a purified control diet with a high starch content and look at bile acid metabolism with a special interest in the ileum.

In the following chapter (chapter 4) the effects of indigestible carbohydrates, in this case soluble fibres, on FGF15/19 signalling in the ileum are investigated.

In chapter 5 we broaden our view and investigate additional effects the HS diet has on the gut-liver axis and additionally investigate if adding soluble fibres to the HS diet can counteract some these effects on the gut-liver axis.

In the last research chapter (chapter 6), we study bile acid metabolism during healthy ageing. In collaboration with the group of Dr Steegenga at Wageningen University small intestinal samples of a 24 months mouse aging study were received. Using these samples, we investigated bile acid metabolism in the small intestine during normal and healthy ageing.

Chapter 2: Materials and Methods

Thesis Britt Blokker

This chapter contains background information about the materials and methods used troughout this thesis. More detailed methods are discussed per chapter.

2.1 Mice and organ collection

For all murine studies male C57B6/J mice were used and mice were under 12 hours of light and dark cycles. Mice studies were either performed at Wageningen University (WUR) prior to the start of this PhD project, or by the PhD candidate at the Disease Modelling Unit (DMU) of the University of East Anglia (UEA). A table at the start of each chapter indicates where the studies were performed, if they were performed by the PhD candidate and if not, what was made available to the PhD candidate. All animal experiments were started at the age of 10-12 weeks, after stabilization of the microbiota (Schloss et al. 2012).

All experimental procedures and protocols performed for this PhD thesis were reviewed and approved by the Animal Welfare and Ethical Review Body and were conducted according to the specifications of the United Kingdom Animals Scientific Procedures Act, 1986.
The animal experiments performed at WUR were in line with the Dutch guidelines. The study designs and the experiments described were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University.

Mice were sacrificed in the fed state in both UEA and WUR studies, unless indicated differently. UEA mice were sacrificed by cardiac puncture, a method in which animals are anesthetized with isoflurane gas and all blood is with drawled from the body via the heart. The small intestine of each mouse was transferred to a cold glass plate and divided into three equal pieces. The content of each piece was subsequently pushed out gently and collected into a separate Eppendorf tube (per part) which is immediately frozen in liquid nitrogen or dry ice. After pushing out the content, each part was cut into three small and three large pieces, as is depicted in figure 2.1. The most proximal large part was collected in a cassette and stored in 10% formalin (during 24 hours) for histology. The three small parts were collected together and put into an Eppendorf tube that was immediately snap-

frozen in liquid nitrogen. These parts were later used for RNA isolation. The remaining two larger pieces were collected in a cryotube which was also snap-frozen in liquid nitrogen immediately. These parts were used for the remaining measurements like protein and bile acid extraction. From the liver of each mouse, two small pieces were collected into a cassette and stored in 10% formalin, while the rest of the liver was collected in a cryotube and snap-frozen in liquid nitrogen. Furthermore, cecum, colon, white adipose tissue (WAT) and brain were collected and snap-frozen in liquid nitrogen. Moments before sacrificing the mice, faeces were collected and frozen on dry ice.

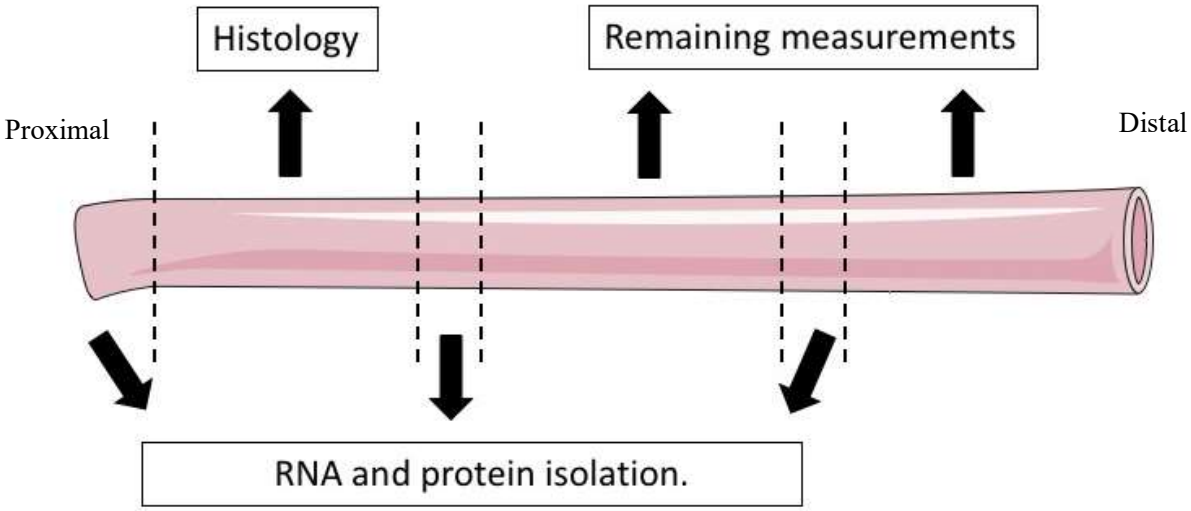

Figure 2.1 Visualization of the sample collection per part of the intestine. The segmentation was performed on each third of the small intestine, the duodenum, jejunum and ileum, at the time of sacrifice.

Collection of the small intestine for the studies performed at WUR were similar, however the SI has been divided up in a variable number of equal pieces. For the IDEAL WUR mice (ageing study, see chapter 6) the SI was divided up into three equal parts, while for the 10 parts WUR mice (see chapter 3 and 5) the SI was divided into 10 equal parts. Additional organs (e.g. liver, cecum, colon, WAT, brain, faeces etc) were not available.

2.3 Treatments

A variety of dietary components and chemicals have been administered to mice in addition to either the chow and/or purified diet. Dietary component and chemicals were either administrated via the drinking water or oral gavage.

2.3.2 Administrating substances via the drinking water

C57B6/J mice drink an average of 5-6 ml of water per day (Bachmanov et al. 2002) and the dose of substances has been based on this consumption. Whenever substances were administrated via the drinking water, drinking water was renewed every three days to avoid breakdown of the product. Additionally, the taste of the substance was taken into account; possible bitter-tasting substances were administrated via oral gavage to avoid dehydration.

2.3.3 Oral gavage

Whenever a substance was less suitable for administration via the drinking water, either because a very specific dose was needed, there was a possible bitter taste or the cost of the substance required was high, oral gavage was the suitable alternative.

Oral gavage was performed by restraining the mouse and via a plastic, flexible catheter (FTP1830 18G x 30mm, Instech Solomon) adding 100 – 200 µL of liquid (usually substance dissolved in PBS) into the stomach. The maximum volume for oral gavage in mice is 20 µL/g BW, however for the comfort of the mice adjusted the concentrations of our substances to oral gavage an average of 5 µL/g BW and a maximum of 10 µL/g BW.

Chapter 3: Glucose uptake into ileal enterocytes increases the expression of the signalling molecule *Fgf15* independent of the microbiota

Thesis Britt Blokker

Name study	Where	Performed by candidate?	Samples available
10 parts study	WUR	No	Microarray data on intestinal tissue
Feeding studies over time	UEA	Yes	All
Antibiotics study	UEA	Yes	All
Antifungal study	UEA	Yes	All
Fasting/refeeding study	UEA	Yes	All
FXR antagonist study	UEA	Yes	All
Cholestyramine study	UEA	Yes	All
Acarbose study	UEA	Yes	All
Glucose studies	UEA	Yes	All

3.1 Introduction

A multitude of metabolic diseases, including diabetes mellitus type 2, have been linked to bile acid metabolism (Thomas et al. 2008; Prawitt, Caron, and Staels 2011; Boesjes and Brufau 2014). Within bile acid metabolism, the role of the metabolic hormone fibroblast growth factor (FGF) 19 and its mouse ortholog *Fgf15* has emerged. Since bile acids are derived from cholesterol and *Fgf15* blocks the production of bile acids via CYP7A1, an increased expression of *Fgf15* reduces the conversion of cholesterol to bile acids. This effect has been linked to elevated blood levels of total and LDL cholesterol in both rodents and humans (Stroeve et al. 2010; Harrison et al. 2018).

Outside of its role in bile acid and cholesterol metabolism, FGF15/19 has shown to activate both protein and glycogen synthesis in the liver (Kir et al. 2011). FGF15/19 is capable of lowering blood glucose levels (Wu et al. 2010) and regulates hepatic glucose metabolism in an insulin independent manner (Potthoff et al. 2011; Kir et al. 2011) Fasted mice treated with FGF15/19 showed a decreased expression of hepatic genes involved in glucose metabolism, regulated by proliferator-activated receptor γ coactivator protein-1α (PGC-1α), of which gene expression is induced by cAMP regulatory element-binding protein (CREB) under fasting conditions (Herzig et al. 2001), showing that FGF15/19 modulates hepatic glucose metabolism by inhibiting the CREB-PGC1a pathway (Potthoff

et al. 2011). FGF15/19 upregulation is known to be a postprandial response that follows the insulin response approximately 60 minutes after the consumption of a meal (Potthoff et al. 2011). The postprandial upregulation of FGF15/19 is hypothesized to be in response to the bile acids reaching the ileum. Interestingly, FGF15/19 drug trials are currently ongoing to investigate its possible use in diabetes mellitus treatment (Degirolamo, Sabbà, and Moschetta 2016). However, a substantial obstacle and concern is the potential carcinogenic effects of FGF15/19. The metabolic hormone induces cell division and has led to the development of hepatic carcinoma in mouse models (Uriarte et al. 2015; Uriarte et al. 2013). Currently, a non-carcinogenic engineered FGF19 analogue, NMG282, is being tested for human treatment (Harrison et al. 2018).

Although it is clear that the signalling molecule FGF15/19 is biologically important, not much is known about the influence of nutrition on its regulation, even though it is widely accepted that FGF15/19 up-regulation is a postprandial response. Additionally, *Fgf15* expression is downregulated in both germ-free and antibiotic treated mice, indicating a link with the gut microbiota (Sayin et al. 2013b; Miyata et al. 2009; Wahlström et al. 2016). This is also shown by the effects the antioxidant tempol that alters ileal FXR activity via alteration in the microbiota (Li et al. 2013). The link between FGF15/19 and the microbiota could suggest dietary regulation, as diet and environment are the main determinants of microbiota composition in both mice (Carmody et al. 2014) and humans (Rothschild et al. 2018).

Because of the regulation of FGF15/19 expression by the microbiota and its newly discovered role in blood glucose regulation, we hypothesized that FGF15/19 might be upregulated by diets high in digestible carbohydrates, leading to increased ileal glucose levels. Here we show that starch intake, and thereby increased glucose concentrations in the ileum, are upregulating *Fgf15* expression in C56BL6/J mice, and that this effect is independent of the microbiota. On long term dietary intervention, this leads to disturbance of bile acid metabolism, increased cholesterol levels and even the development of hepatic carcinoma in mice.

3.2 Materials and methods

3.2.1 Murine studies

C56BL6/J mice bred in the DMU of the UEA were used for all murine studies (except WUR study, see below), starting at the age of 10-12 weeks, with a group size of minimum 5 and maximum 10 mice. At the end of the experiments mice were sedated with a mixture of 4% isoflurane (IsoFlo®, Abbott, ND), nitrous oxide (70%) and oxygen (30%) and blood samples were collected via cardiac puncture. The small intestine was collected and arranged in three equal parts, of which the last part was identified as the ileum. Additionally, the liver was collected. All samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis or fixed in 10% formalin (include if you show histology).

All experimental procedures and protocols used in these studies were reviewed and approved by the Animal Welfare and Ethical Review Body and were conducted according to the specifications of the United Kingdom Animals Scientific Procedures Act, 1986.

The WUR study has been performed at the Nutrition, Metabolism and Genomics group of Wageningen University (WUR) in the Netherlands, previous to me commencing my PhD. Here the small intestine was divided into 10 equal parts on which microarrays (Affymetrix GeneChip Mouse Gene 1.1 ST array) were performed. Our group consequently analysed the data using Microsoft Excel.

3.2.2 Diets

Mice were fed either a standard chow diet (RM3-P, Special Diets Services, UK), a HS purified diet (D12450H, Research Diets, USA) for the indicated duration per experiment. WUR mice were fed a standard chow, a HS (D12450H) diet or a high fat (HF, 45 energy percent (En%) fat, D12451, Research Diets) diet for two weeks, however in the WUR diets lard is replaced by palm oil as a fat source. See table 3.1 for a description of the diets used.

Table 3.1 Dietary composition per kilogram of diet. The high starch (HS) diet contains more starch and sucrose and less fibre compared to the chow diet. In the purified diet (HS and high fat (HF) from Research Diets) starch is exchanged for fat.

	Chow diet SDS diets RM3	HS diet D12450H	HF diet D12451
Energy content	3.35 kcal/g	3.82 kcal/g	4.4 kcal/g
Protein	27.28 En%	20 En%	20 En%
Carbohydrate	61.24 En%	70 En%	35 En%
Fat	11.48 En%	10 En%	45 En%
Corn Starch (g)	338.8	452.2	72.8
Maltodextrin (g)	0	75	100
Sucrose (g)	43.7	172.8	172.8
Dietary fibre (g)	161.5 Mix of soluble and insoluble	50 Cellulose only	50 Cellulose only
Vitamins See appendix 1	Unknown mix	1 g V10001C	1 g V10001C
Minerals See appendix 1	Unknown mix	50 g S10026B	50 g S10026B

3.2.3 Intervention experiments

3.2.3.1 Studies performed at WUR

As explained in Chapter 2, some of the results presented in this work were generated from samples of studies that were performed at WUR. Although these studies were not performed by the PhD candidate, a short summary of the methods used are explained below for the purpose of disclosing all information.

10 Parts study

Male C57B6/J mice were fed a standard chow, a low fat (LF, 10 En%) fat) or a high fat (HF, 45En% fat) diet for two weeks (Research Diets, see table 2.1). At the end of the two weeks mice were sacrificed in the fed state and the small intestine was transported to a

cold glass plate. Subsequently, the intestine was cut into 10 equal pieces which were opened and rinsed in phosphate buffered saline (PBS). The cells were scraped out, and RNA was isolated and converted into cDNA. A microarray (Affymetrix GeneChip Mouse Gene 1.1 ST array) was performed at the WUR and data was analysed by our group as outlined in chapter 2.4.

3.2.3.2 Studies performed at UEA / Quadram Institute

The studies outlined below were performed by the PhD Candidate in the DMU of the UEA and samples were analysed at the Quadram Institute. Body weight and food intake was measured once or twice a week for all experiments. Group size is between 5 to 10 mice per group for each experiment. Mice were transferred to a clean cage every week, unless stated otherwise, and were sacrificed in the fed state, unless stated otherwise (see below).

Feeding studies over time

Mice on a chow background diet were fed the chow or HS diet for a duration of 3 days, 1 weeks, 2 weeks, 8 weeks or 6 months before sacrifice.

Antibiotics study

Mice on a chow background were switched to a clean cage and either remained on a chow diet or switched to the HS diet for 1 week. During this week mice were oral gavaged once daily with a cocktail of vancomycin (50mg/kg BW, Sigma-Aldrich, 94747), Neomycin (100mg/kg BW, Sigma-Aldrich, N6386), Metronidazol (100mg/kg BW, Sigma-Aldrich, M1547) in 0.9% saline. Additionally, mice received ampicillin (1g/L, Sigma-Aldrich, A0166) via drinking water. Mice were transferred to a clean cage every other day for the duration of this experiment. Similar protocol used in (Zarrinpar et al. 2018).

Antifungal study

Mice on a chow background were switched to a clean cage and either remained on a chow diet or switched to the HS diet for 1 week. During this week mice received fluconazole (Sigma-Aldrich, F8929) at a concentration of 0.5mg/ml in their drinking water. Additionally, there was a group of mice on both the chow and the HS diet that received

both the antibiotics treatment explained above and this antifungal treatment simultaneously during one week.

Fasting/refeeding study

Mice on a chow background were fasted overnight for a total of 12 hours (8pm-8am). At 8am, mice were re-exposed to food, either the chow or the HS diet for a total of 3 hours before sacrifice.

FXR antagonist study

Mice were fed either the chow or HS diet and received the FXR antagonist Guggulsterone (Tocris, Bio-Techne, UK) via a daily oral gavage. Guggulsterone was given at a dose of 20 mg/kg BW dissolved in PBS.

Cholestyramine study

Cholestyramine is a bile acid chelator and was administered twice daily via oral gavage (AM and PM) for the duration of one week to mice on either the chow or HS diet. Mice received a dose of 500mg/kg BW dissolved in PBS each time.

Acarbose study

To inhibit starch digestion, the competitive inhibitor acarbose (Sigma-Aldrich, A8980) was added to the drinking water of mice on both the chow and HS diet for two weeks. Acarbose was added to the drinking water because of it competitive nature, so that the mice received the acarbose throughout the day and night when they were also consuming their food. Acarbose was added to the drinking water at a concentration of 0.31g/L to reach a dose of approximately 50mg/kg BW/day.

Glucose studies

Glucose studies were based on glucose tolerance tests, where mice often receive a dose of D-Glucose (Sigma-Aldrich, G8270) at 2g/kg BW dissolved in PBS by either oral gavage or Intraperitoneal (IP) injection.

3.2.4 *In vivo* measurements

Transit time

Transit time from stomach to faeces was determined by Evans Blue. The Evans Blue dye (Sigma-Aldrich, E2129) was mixes with the original diet (either chow or HS diet) and PBS at a concentration of 5% Evans Blue, 10% chow/HS diet and 85% PBS. Mice were oral gavaged with 0.1mL of this semiliquid and overall transit time was determined by the time the first blue dye was determined in the faeces. Transit time was determined in mice that has been on the chow and HS diet for 12 weeks.

Glucose measurements

While most glucose was measured post-mortem, during the glucose studies some of the glucose measurements were performed *in vivo*. Blood for *in vivo* glucose measurements was sampled via a tail prick with a 23G needle. Glucose was consequently measured using a glucose meter (AlphaTRAK 2 Abbott).

3.2.5 Gene expression

RNA of liver and intestinal scraping samples was isolated. Samples were homogenised in 1 ml Trizol (Qiazol, Qiagen, UK) for 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France). After homogenization samples were transferred to a new Eppendorf tube and 200 µl Chloroform was added. Samples were centrifuged for 15 minutes at 12.000 RCF 4˚C and the clear layer was transferred to a new collection tube. Iso-propanol was added and after mixing, resting for 5-10 minutes and centrifugation for 10 minutes at 12.000 RCF 4˚C an RNA pellet was left. The pellet was washed in 85% ethanol and dissolved in 100 µl RNase Free water. RNA concentration was measured with a Nanodrop (Thermo Scientific, Wilmington, USA). cDNA was synthesized from 2 µg of RNA which was first treated with DNase (DNase kit, Invitrogen, 18068-015) and subsequently reverse transcription was performed. A quantitative polymerase chain reaction (Q-PCR) was performed using SYBR green master mix (Applied Biosystems, Thermo Fisher Scientific, UK) according to the producer's instructions. Reactions were performed using 384 wells (Applied Biosystems, Thermo Fisher Scientific, UK) using

5µL of SYBR green and primer mix (for a list of the primers used, see table 3.2) and 1.5µL of cDNA. The reaction was initialized at 50°C for 5 minutes and 95°C for 2 minutes, after which 40 cycles of denaturation (95°C 15 seconds) and annealing/extension (59°C 1 min) were performed. Afterwards, a melting curve was created and checked for a single product per gene. ΔCT values were calculated by subtracting the CT value of the housekeeping gene TBP from the gene CT value. ΔΔCT was calculated relative to control samples (Livak and Schmittgen 2001).

Table 3.2 Primers used for q-PCR measurements in small intestine and liver.

Gene	Forward	Reverse
TBP (hk)	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT
GAPDH (hk)	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
FXR	GGCCTCTGGGTACCACTACA	AAGAAACATGGCCTCCACTG
OSTA	TTGTGATCAACCGCATTTGT	CTCCTCAAGCCTCCAGTGTC
ASBT	TGGAATGCAGAACACTCAGC	GCAAAGACGAGCTGGAAAAC
FABP6	CACCATTGGCAAAGAATGTG	AACTTGTCACCCACGACCTC
SHP	TCTGCAGGTCGTCCGACTATT	AGGCAGTGGCTGTGAGATGC
FGF15	CAGTCTTCCTCCGAGTAGCG	TGAAGACGATTGCCATCAAG
CYP7A1	GAGCGCTGTCTGGGTCACGG	GCCAGCCTTTCCCGGGCTTT
CYP27A1	TGTGGACAACCTCCTTTGGG	CCATAGGTGAGGCCCTTGTG
CYP8B1	TTGCAAATGCTGCCTCAACC	TAACAGTCGCACACATGGCT

Selected samples went for microarray analysis. After RNA isolation as described before, samples were send to WUR for microarray analysis. At WUR RNA quality was assessed with a Bioanalyzer (Aligent 2100 Bioanalyzer, Santa Clara, USA), and microarrays were performed using the Mouse gene Chip 1.1 array from Affymetrix (Thermo Fisher Scientific, Santa Clara, USA). After normalization process, ~22 k genes were included in the data set. The limma R library was used to analyse the significant differential expression between the diets. Log2FC of 1.5 and a p-value of 0.05 were used as cut-off points and the rate discovery false for the p-value was calculated. Ingenuity Pathways

Analysis (IPA) web-based software application was used to analyse, integrate and interpret the data

3.2.6 Histology

From each UEA mouse, a small part of the liver and a piece of each of the three parts of the small intestine was collected in 10% formalin. 24 hours after collection, these samples were transferred into 50% ethanol and within a week were processed on the Leica ASP 300, program overnight no formalin. The samples were embedded in wax and cut into 5µm sections. Sections were stained with a haematoxylin and eosin (H&E) staining and pictures were taken with an Olympus BX60 microscope.

3.2.7 Western blot

Whole cell extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% non-fat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was washed once with TBST and incubated with antibodies against FXR (1:1000 dilution, Santa-Cruz, sc-25309), GAPDH (1:5000 dilution, Abcam, AB8245) at 4 °C for 12 h. Membranes were washed three times for 10 min and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 2 h. Blots were washed with TBST three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocols

3.2.8 Bile acid measurements

As no bile acid measurements were performed in house at the time of arrival, we set up our own method in collaboration with the metabolomics unit of the Quadram Institute. A special thanks go to Mark Philo, who was a tremendous help in this process. Bile acids were measured in the scrapings and content of the three different parts of the small intestine, liver, colon faeces and serum. Here for, 50 mg (liver), 25 mg (intestine) or 50 µL (serum) of tissue was homogenised in 1 ml of cold 70% methanol for 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France). After centrifugation (5 min,

3000g, 4˚C) the supernatant was transferred to a new collection tube and MeOH content was removed by rotary evaporation (70 min, 50˚C). Volume was restored to 1ml by adding 5% MeOH. To clean up the samples they were loaded onto Waters OASIS PRIME HLB 1 30mg SPE cartridges, washed with 5% MeOH and eluted in 500 µl 100% MeOH.
Internal standards were added at the following time points: before homogenisation, before rotary evaporation, before loading onto cartridges, after elusion.
Cleaned-up extracts were analysed using HPLC – mass spectrometry operated in multiple reaction monitoring (MRM) mode.
Each sample (5 µl) was analysed using an Agilent 1260 binary HPLC couples to an AB sciex 4000 QTrap triple quadrupole mass spectrometer. HPLC was achieved using a binary gradient of solvent A (Water + 5mM Ammonium Ac + 0.012% Formic Acid) and solvent B (Methanol + 5 mM Ammonium Ac + 0.012% Formic Acid) at a constant flow rate of 600 µl/min. Separation was made using a Supelco Ascentis Express C18 150 x 4.6, 2.7µm column maintained at 40˚C. Injection was made at 50% B and held for 2 min, ramped to 95%B at 20 min and held until 24 minutes. The column equilibrated to initial conditions for 5 minutes.
The mass spectrometer was operated in electrospray negative mode with capillary voltage of -4500V at 550˚C. Instrument specific gas flow rates were 25ml/min curtain gas, GS1: 40ml/min and GS2: 50ml/min.
See appendix 2 for liquid chromatography-mass spectrometry (LC-MS) conditions and mass fragmentation monitoring values. Quantification was applied using Analyst 1.6.2 software to integrate detected peak areas relative to the deuterated internal standards.

3.2.9 Microbiota analysis

3.2.9.1 microbial DNA isolation from SI content

The content of each part of the small intestine was gently pushed out of the intestine at the time of sacrifice and rinsed out using 200µ of PBS. 150µL of this was used to isolate microbial DNA from. In collaboration with the Hall lab (Quadram Institute, Norwich Research Park, UK), for DNA isolation we used the Qiagen DNA mini kit following an improved protocol with additional steps to ensure breakage of all bacterial samples kindly provided by Dr. M Lawson. Briefly, the samples were homogenised using glass beads for

4x 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France) and heated to 95°C for 5 minutes in the middle. Additionally, samples were incubated with a lysis buffer containing 20mg/ml lysozyme after which the homogenising was repeated. Consequently, DNA was isolated using the Qiagen DNA mini kit following instruction from the producer. DNA quantity was assessed using both a nanodrop and a Qubit reader.

3.2.9.2 16S sequencing of the SI microbiota

A minimum of 50 ng of DNA was sent to the Earlham Institute (Norwich Research Park, UK) for quality assessment and 16S sequencing using the Illumina platform. Results were further analysed in collaboration with Wiktor Jurkoswki, PhD and Sarah Bastkoswki, PhD from the Earlham Institute.

The microbiome composition was established using QIIME version 1.9.0 (Caporaso et al. 2010). Paired reads were merged during the Quantitative Insights into Microbial Ecology Qiime 1.9.0 illumina workflow employed on a high performance computing cluster. The mapping file includes the forward and reverse linker primer sequence and was used for demultiplexing. Demultiplexing, quality filter and mapping file validation was carried out as described in the Qiime illumina workflow manual. Operational Taxonomic Units (OTUs) were picked within Qiime using the closed-reference OTU picking protocol with the RDP classifier by searching reads against the Greengenes database version gg_13_8 to the 97% level, and reads were aligned with PyNAST. A representative sequence tree was gained after clustering using Uclust version 1.2.22.

We calculated richness as defined through Chao1 (Chao A, (2000)) and diversity as defined by Shannon (Shannon C, (1948)) using the R package Phyloseq (version 1.18.0) (McMurdie P et al., (2013)). The Shapiro-Wilk test was used to assess normality and showed that the alpha diversity values are not normally distributed. We tested if diets had a significant impact on the mouse microbiomes alpha diversity using the Kruskal-Wallis test. A modified one way ANOVA as implemented in lmPerm (version 2.1.0, Wheeler R E, (2010)) was employed, which uses permutation tests instead of normal theory test, and was used to test for effects diet as covariates.

We used the fitZig function of the R package Metagenomeseq (Paulson et al. 2013) to establish significant differences in abundances of OTUs between groupings including covariates. We considered OTUs that were present in at least 25% of all samples.

3.2.9.3 Quantification of the microbiota using Q-PCR

Quantification of the microbiota was performed to analyse the effectiveness of administrating antibiotics. A standard curve was created by growing E. Coli until an optical density of 2 as measured on a spectrophotometer. Microbial DNA was isolated from this growth medium using the same method as described above and diluted into a standard curve. Total 16S DNA was quantified by q-PCR using the Eub primers (table 3.3), relating the quantifiable samples to the produced standard curve.

Table 3.3 Primers used for microbial DNA.

Microbial DNA	Forward	Reverse
Eub	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG
Bacteroides	GGARCATGTGGTTTAATTCGATGAT	AGCTGACGACAACCATGCAG
Firmicutes	GGAGYATGTGGTTTAATTCGAAGCA	AGCTGACGACAACCATGCAC

3.2.10 Glucose and starch quantification in intestinal content

Glucose in the content of the ileum was determined in mice that were oral gavaged with a high dose of glucose to determine if the glucose reached the ileum. Glucose quantification was performed using a glucose assay kit (Sigma-Aldrich GAG020). 20mg of content was homogenized in 500 µL deionized water, heated at 70°C for 15 minutes and centrifuged for 10 minutes at 3000g. The supernatant was used in the glucose assay kit using the manufactures instructions.

Starch was quantified in the content of the ileum and caecum in order to determine starch digestion higher up in the small intestine using the total starch assay (Megazyme, K-TSTA-50A). 20mg of content was homogenized in 1 mL of 80% Ethanol and centrifuged for 10 minutes at 1800g. After wash steps, starch was digested by alpha-amylase according

to the manufactures protocol. Finally, glucose is measured in an absorbance plate reader at 510 nm.

3.2.11 Serum and liver measurements

Transaminases Alanine transaminase (ALT) and Aspartate transaminase (AST) were tested in the serum using a routine colorimetric ILAB 650 assay to determine normal liver function.

Cholesterol was determined in the serum using the corresponding liquicolor kits (Human GmbH, Germany) following the manufactures protocol.

Cholesterol and triglycerides were also analysed in the livers using the same kits. Proceeding this, the lipid fraction was extracted following an adjusted version of the Bligh and Dyer method (Bligh and Dyer 1959). In short, 50 mg of liver tissue was homogenized in 200 µL chloroform and 400 µL methanol. 200 µL distilled water was added and the sample was homogenized again after which the sample was centrifuged for 10 minutes at 3000g. The lower layer was subsequently transferred to a clean Eppendorf tubes and dried overnight. The dried pellet was dissolved in 250 µL 2% Triton X-100 and this was used to perform the colorimetric assay.

3.2.12 Statistics

Statistics have been performed using Graphpad Prism. The statistical tests used are indicated in each figure legend. Power calculations were based on Fgf15 results observed in the 10 parts study and led to a minimum of 5 mice per group being used in each experiment (indicated per figure). Analysis of variance (ANOVA) provides a statistical test of whether the means of groups are equal. When groups were compared over time, a 2-way ANOVA with Bonferroni post-hoc test was used. When three or more groups were compared at a single time point, a 1-way ANOVA was used with Bonferroni post-hoc. Lastly, when two groups were compared at one time point, an unpaired t-test was used to test for significance. $p<0.05$ was considered overall as statistically significant and significance was indicated as ***= $p<0.001$ **= $p<0.01$ *= $p<0.05$.

3.3 Results

To investigate the effects of diets on bile acid metabolism and the regulation of the signalling molecule *Fgf15*, a standard chow diet and a purified HS diet were compared over time. As expected, no changes in body weight or food intake were observed between the two groups over a six-month period (figure 3.1A and B). More information about the composition of the diets can be found in table 3.1.

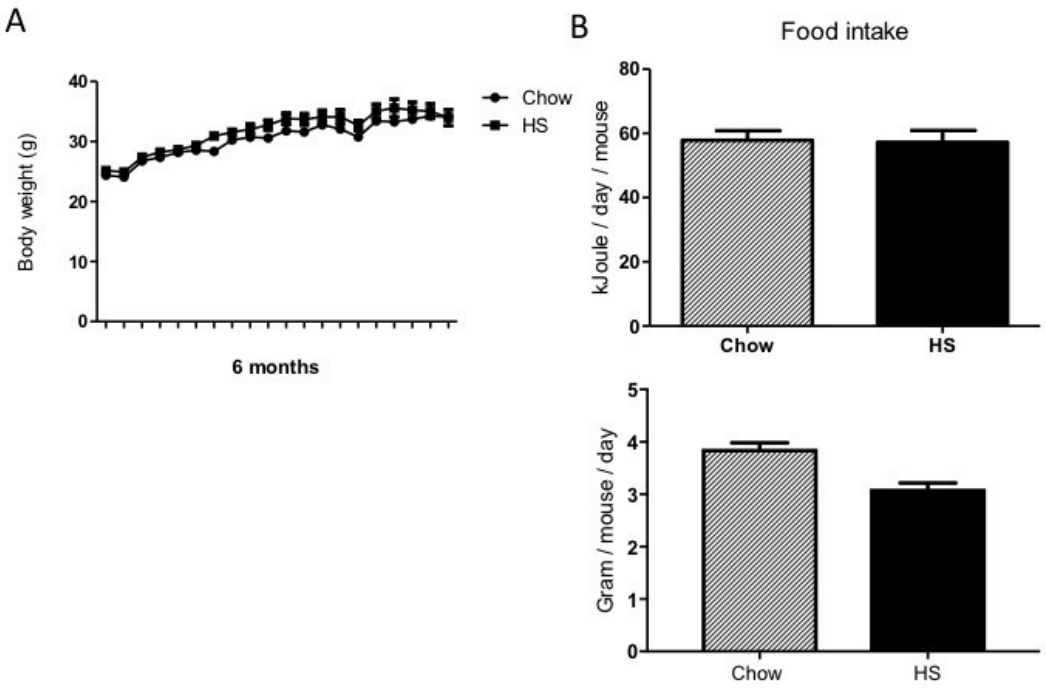

Figure 3.1 The high starch (HS) diet does not lead to changes in body weight in mice. Mice were fed a standard chow diet or a purified HS diet for up to six months. During this time, no differences in either body weight (A) or calorie intake (B) were observed. *Significance was tested using 2-way ANOVA with Bonferroni post-hoc test (A) and an unpaired t-test (B)*

3.3.1 A HS diet increased the expression of the signalling molecule *Fgf15* in the ileum independent of bile acids

Despite the lack of difference in body weight, aspects of bile acid metabolism were found to be differentially regulated in the two diet groups. The most striking change was a 2-6 fold increase in the expression of the signalling molecule *Fgf15* in the HS fed mice. *Fgf15* expression was increased on all measured time-points (3 days to 6 months, figure 3.2A) and this difference in gene expression was confirmed in an independent study performed at Wageningen University (WUR, NL). The WUR study was performed with mice on a 2 week-intervention with chow, HS and HF-diets. Both the HF and HS diet are purified diets, the only difference being that a large part of the starch (379.4g / 84%) is replaced by saturated fat in the HF diet. Changes in gene expression have been studied by using whole genome microarrays over the course of the small intestine. *Fgf15* expression showed an expected increase toward the ileal part of the small intestine in chow fed mice and showed also a pronounced upregulation on the HS diet compared to the chow diet (figure 3.2B). The HF-fed mice show *Fgf15* expression pattern more similar to the chow group, suggesting that potentially the amount of starch may play a role in ileal *Fgf15* expression levels.

Since bile acids are known to be the major regulator of *Fgf15* expression, bile acids present in the ileal tissue at the time of sacrifice were analysed via HPLC and LC-MS. No differences in total bile acids and no consistent differences in individual bile acids were observed between the two diets (figure 3.2C, D and table 3.4). The minor differences in individual bile acids observed were not consistent over the different time point and would not easily explain the difference in *Fgf15* expression. Overall, most differences would suggest higher FXR activity on the chow diet, which would lead to the opposite effect of that observed in the current study. For example, after 3 days of feeding tauro conjugated beta muricholic acid (T-b-MCA) is significantly higher on the HS diet compared to the chow diet, while it has been known to be a FXR antagonist (Sayin et al. 2013b; Gonzalez et al. 2017) and should lead to a decrease in *Fgf15* expression. Taken together, these results indicate that dietary starch increases the expression of *Fgf15* in the ileum independent of changes in bile acids.

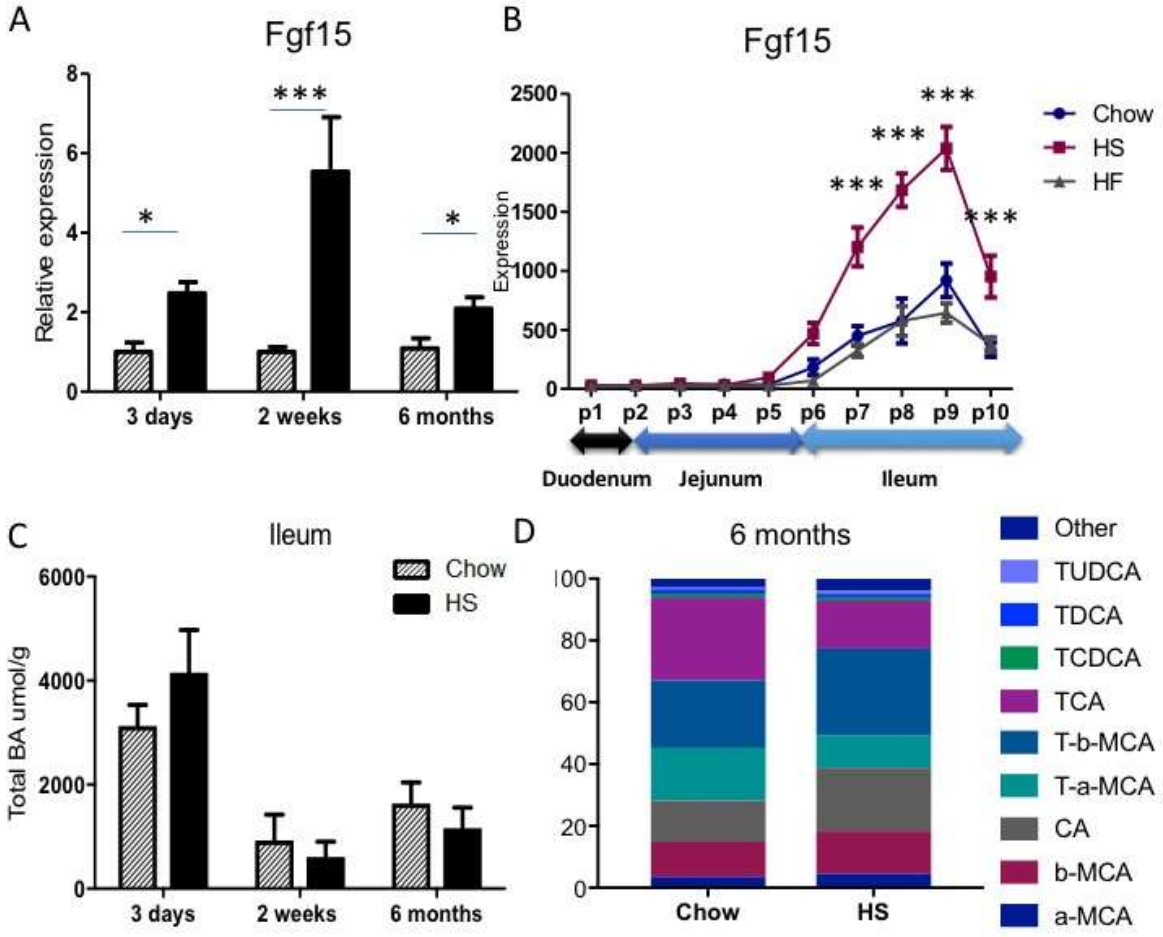

Figure 3.2 A high starch diet increases the expression of *Fgf15* in two independent studies while no differences in ileal bile acids are observed. *Fgf15* expression in the ileum is increased in mice on a HS diet at all measured time points, from 3 days to 6 months (A). An independent study performed at Wageningen University shows *Fgf15* expression levels over the course of the small intestine, confirming the increase of Fgf15 of mice on a HS diet in the ileum compared to both a control chow diet and a high fat (HF) diet (B). Bile acids were measured in ileal tissue and show no differences in total bile acids after 3 days, 2 weeks and 6 months of feeding (C) and the major bile acid groups after 6 months of feeding the chow versus HS diet (D). *Significance was tested using or 2-way ANOVA with Bonferroni post-hoc test between chow and the test diets (***= p<0.001, *= p<0.05) n≤5 for all groups*

Table 3.4 Ileal bile acid profiles show no consistent alteration between chow and HS fed mice. Bile acid were quantified in ileal tissue by HPLC and are given in µmol/g tissue after 3 days, 2 weeks and 6 months of feeding the chow and HS diet. *Significance was calculated using a 2-way ANOVA with Bonferroni post-hoc test between chow and HS (***= p<0.001, **= p<0.01, *= p<0.05) n≤5 for all groups Color coding indicated conjugation, white being tauro-conjugated, light grey being glyco-conjugated and dark grey being unconjugated bile acids*

	3 days			2 weeks			6 months		
	Chow	HS	P value	Chow	HS	P value	Chow	HS	P value
T-a-MCA	825.56±135.42	1242±612.10	*	424.92±367.81	348.66±331.05	ns	327.20±230.35	179.00±187.33	ns
T-b-MCA	640.39±135.42	1242.67±612.10	**	293.83±288.63	280.79±239.79	ns	462.03±352.89	377.05±331.60	ns
TCA	508.92±144.08	847.44±373.00	ns	247.57±191.85	104.14±111.83	ns	562.92±422.49	128.01±114.11	***
TCDCA	47.49±33.73	149.58±122.00	ns	13.78±13.11	9.62±12.70	ns	27.46±24.96	10.01±11.09	ns
TDCA	31.59±33.73	142.00±122.00	ns	7.54±6.56	3.30±4.71	ns	26.70±31.13	11.72±14.61	ns
TLCA	0.32±0.10	1.33±1.83	ns	0.09±0.10	0.06±0.11	ns	0.00±0.00	0.00±0.00	ns
GCA	18.08±8.35	6.33±4.46	ns	4.75±3.36	2.63±1.77	ns	4.46±4.11	0.94±1.05	ns
GCDCA	0.24±0.23	0.09±0.11	ns	0.00±0.00	0.00±0.00	ns	0.00±0.00	0.00±0.00	ns
GDCA	0.31±0.18	0.13±0.16	ns	0.00±0.00	0.00±0.00	ns	0.00±0.00	0.00±0.00	ns
GLCA	0.09±0.15	0.02±0.04	ns	0.00±0.00	0.00±0.00	ns	0.00±0.00	0.00±0.00	ns
a-MCA	190.94±112.18	56.39±37.53	ns	154.76±124.88	67.67±51.43	ns	35.66±23.38	34.17±35.08	ns
b-MCA	305.55±61.21	51.12±34.17	ns	118.01±101.22	67.24±55.08	ns	174.95±150.87	133.73±127.63	ns
CA	426.58±104.13	266.92±146.32	ns	273.36±194.98	201.83±167.58	ns	226.83±188.54	243.21±234.61	ns
CDCA	0.58±0.42	0.20±0.15	ns	0.25±0.12	0.10±0.07	ns	4.28±3.76	1.75±1.70	ns
DCA	48.82±20.49	19.29±13.22	ns	19.01±11.74	8.25±7.27	ns	9.76±6.96	6.08±6.86	ns
UDCA	16.31±6.07	5.39±5.09	ns	4.55±3.42	4.16±6.31	ns	2.69±2.21	2.09±2.47	ns
LCA	2.50±2.86	1.05±1.04	ns	1.18±0.40	0.32±0.39	ns	0.00±0.00	0.00±0.00	ns
MCA	5.74±2.35	1.21±0.78	ns	1.84±1.32	0.32±0.39	ns	0.00±0.00	0.00±0.00	ns

3.3.2 Dietary regulation of *Fgf15* modulates hepatic bile acid and cholesterol metabolism

After production, the signalling molecule *Fgf15* is excreted into the portal vein and is transported to the liver to block *de novo* bile acid production via blocking *Cyp7a1* (figure 3.3A). To test the biological effects of the increased *Fgf15* expression on the HS diets *Cyp7a1* gene expression was measured in the liver. In line with the known effects of *Fgf15*, *Cyp7a1* expression was decreased on all time point in the HS fed mice and this effect increased in significance over time (figure 3.3B). Hence, starch-mediated dietary regulation of *Fgf15* is capable of affecting hepatic bile acids metabolism.

Cyp7a1 converts cholesterol in bile acids, which for the bile acids that escape reabsorption in the ileum, is one of the only known ways of cholesterol excretion in mammals. Since the previously observed decrease in *Cyp7a1* expression could influence cholesterol metabolism, both serum cholesterol and hepatic cholesterol were quantified. An increase in both hepatic and circulating cholesterol levels, that became significant in the long term

was observed (figure 3.3C). This indicates a HS diet can increase the expression of the signalling molecule FGF15 to an extent that it influences both bile acid and cholesterol metabolism.

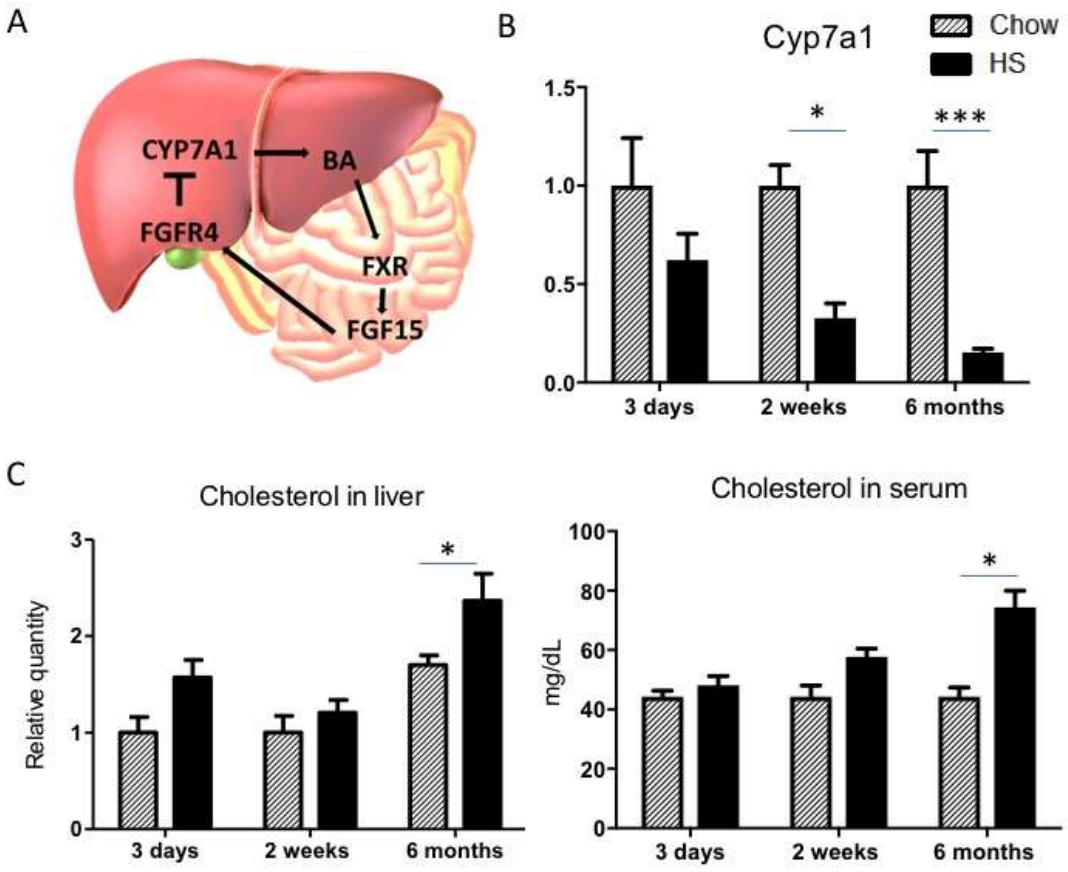

Figure 3.3 A HS diet decreases *Cyp7a1* expression and increases cholesterol levels. FGF15 produced in the ileum travels to the liver to block *Cyp7a1* expression (A), which explains the decrease in *Cyp7a1* expression observed in mice fed the HS diet (B). Cholesterol level were measured in both the serum and the liver (C). *Significance was tested using 2-way ANOVA with Bonferroni post-hoc test between chow and HS diet (***= p<0.001, *= p<0.05) n≤5 for all groups*

3.3.3 Involvement of the microbiota

It has been shown previously that the colonic microbiota is vastly different between a standard chow and a purified HS diet showing both a decrease in diversity and a large alteration in the kind of bacteria present (Dalby et al. 2017). Since the peak abundance of FGF15/19 expression is in the ileum, we decided to sequence the small intestinal microbiota to investigate its potential role in the regulation of Fgf15 expression under HS diet conditions. Microbial DNA was isolated from the content of the small intestine after two weeks of feeding and the 16S gene loci was sequenced. As expected, major differences in the small intestinal microbiota were identified, with switch from *Bacteroidetes* being the dominant phyla to *Firmicutes* being dominant in the HS fed mice (figure 3.4A). Additionally, microbial diversity was found to be lower in the HS fed mice as calculated by Shannon diversity (figure 3.4B).

To investigate causality of the differences in microbiota in the dietary regulation of *Fgf15*, mice were treated with a cocktail of broad spectrum of antibiotics to deplete the number of bacteria significantly in the intestine. Additionally, a group of mice was treated with the antifungal agent fluconazole (0.5mg/ml in drinking water) to abolish the fungal part of the microbiome, and another group of mice received both treatments in combination. Subsequently, *Fgf15* expression was measured in the ileum and showed increased expression in all of the HS fed mice independent of the treatment (figure 3.4C). In line with literature (Miyata et al. 2009), *Fgf15* expression was found to be significantly decreased after antibiotic treatment in chow fed mice (10 fold decrease, $p=0.03$ for chow versus chow+ab, figure 3.4C). To confirm that the antibiotics treatment successfully decreased the number of bacteria in the ileum, microbiota was quantified via q-PCR showing a significant depletion in both diet groups after antibiotics treatment (figure 3.4D). Furthermore, the microbiota changes of mice fed the HS diet are very similar to the microbiota changes in mice fed the HF diet (data not shown, similar results published (Dalby et al. 2017)), while *Fgf15* expression is only upregulated on the HS diet and not the HF diet. This underlines the independency on the microbiota for the purified diets.

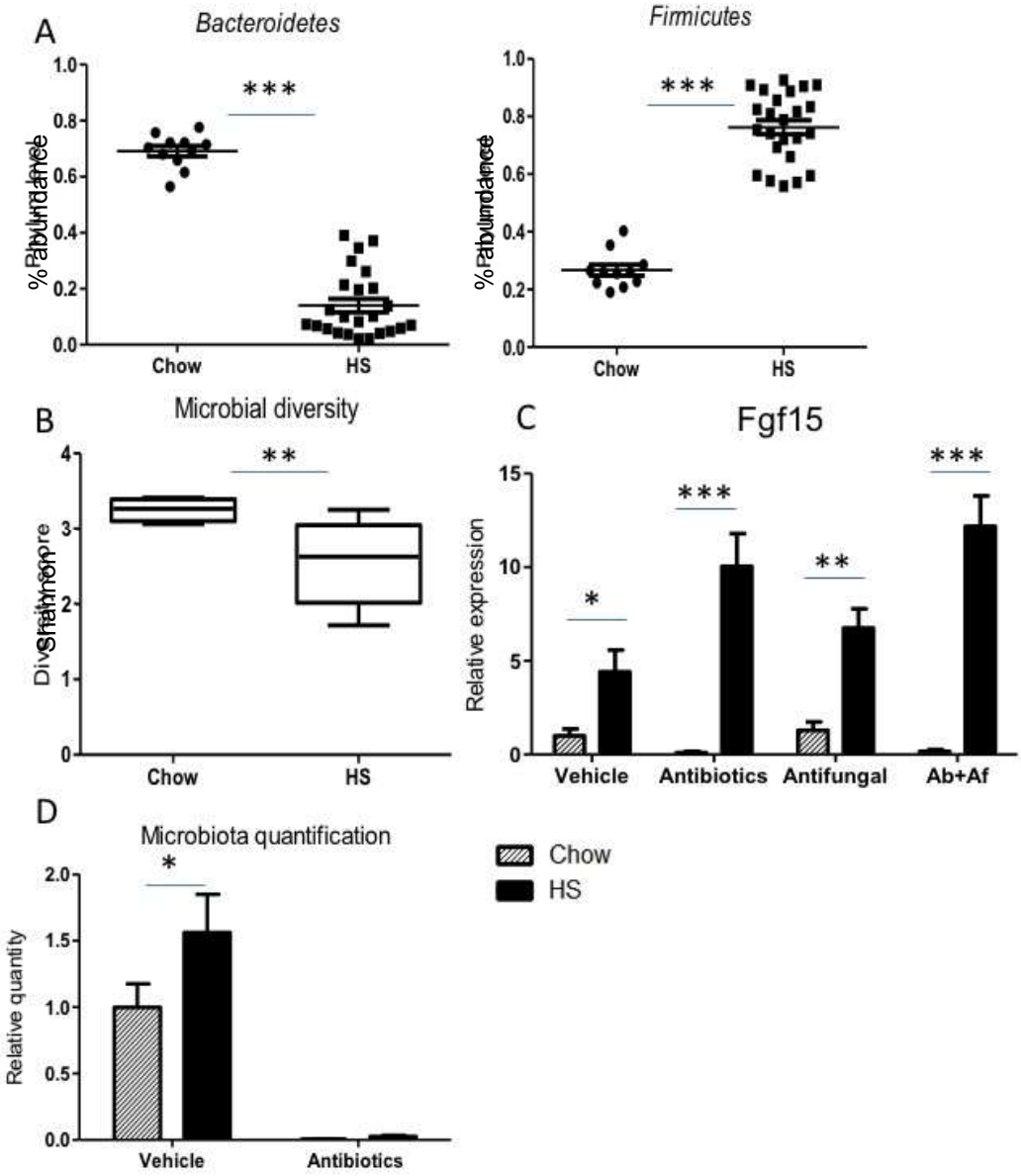

Figure 3.4 The small intestinal microbiota is vastly different between the chow and HS diet but is not causal in the observed changes in *Fgf15* expression. Microbial DNA was isolated from the content of the small intestine and showed a switch from *Bacteroidetes* to *Firmicutes* as the predominant phyla in HS fed mice (A). Additionally, microbial diversity was found to be decreased (B). Mice were treated with a cocktail of broad spectrum antibiotics, fluconazole as an antifungal agent or a combination of both treatments to test the causality of the microbiota on the different Fgf15 expression. None of the treatments decreased *Fgf15* expression on the HS diet (C). Microbiota was quantified to test the effectiveness of the antibiotic treatments (D). *Significance was tested using an unpaired t-test (A, B, C) or a 2-way ANOVA with Bonferroni post-hoc test (C, D) between chow and HS diet (** = p<0.001, **= p<0.01, *= p<0.05) n≤5 for all groups*

3.3.4 Direct effects of HS diet on FGF15 expression

The potential exclusion of causality of the microbiota in the starch regulation of *Fgf15* insinuated a direct effect of components of the HS diet. To investigate this, mice were exposed to the diets for a very short time. 12 week old mice that had been receiving the chow diet since weaning were fasted for 12 hours overnight and consequently refed with either the chow or HS diet for 3 hours before sacrifice, in order to investigate the direct effects of the diet on *Fgf15* expression. The overnight fast ensured food consumption during these 3 hours and digested food was found in the small intestine after sacrifice. Anticipating this experiment, transit time from stomach to faeces was determined to be around 2.5 hours and not significantly different between diet groups (figure 3.5A) indicating that 3 hours of refeeding the diets will allow the diets to pass through the intestine. Others have used labelled activated charcoal to show that passage through the small intestine takes place in approximally 1 hour in mice (Padmanabhan et al. 2013). After refeeding, a trend ($p=0.06$) towards higher *Fgf15* expression in mice fed the HS diet was observed indicating a direct effect of the HS diet on *Fgf15* expression (figure 3.5B).
To investigate the amount of starch reaching the ileum, both starch (figure 3.5C) and glucose levels (figure 3.5D) were quantified in the ileum and were found to be significantly higher in mice fed the HS diet compared to chow fed mice. A positive correlation was observed between both starch and glucose with *Fgf15* (figure 3.5E) indicating that starch reaching the ileum has a direct effect on *Fgf15* expression.

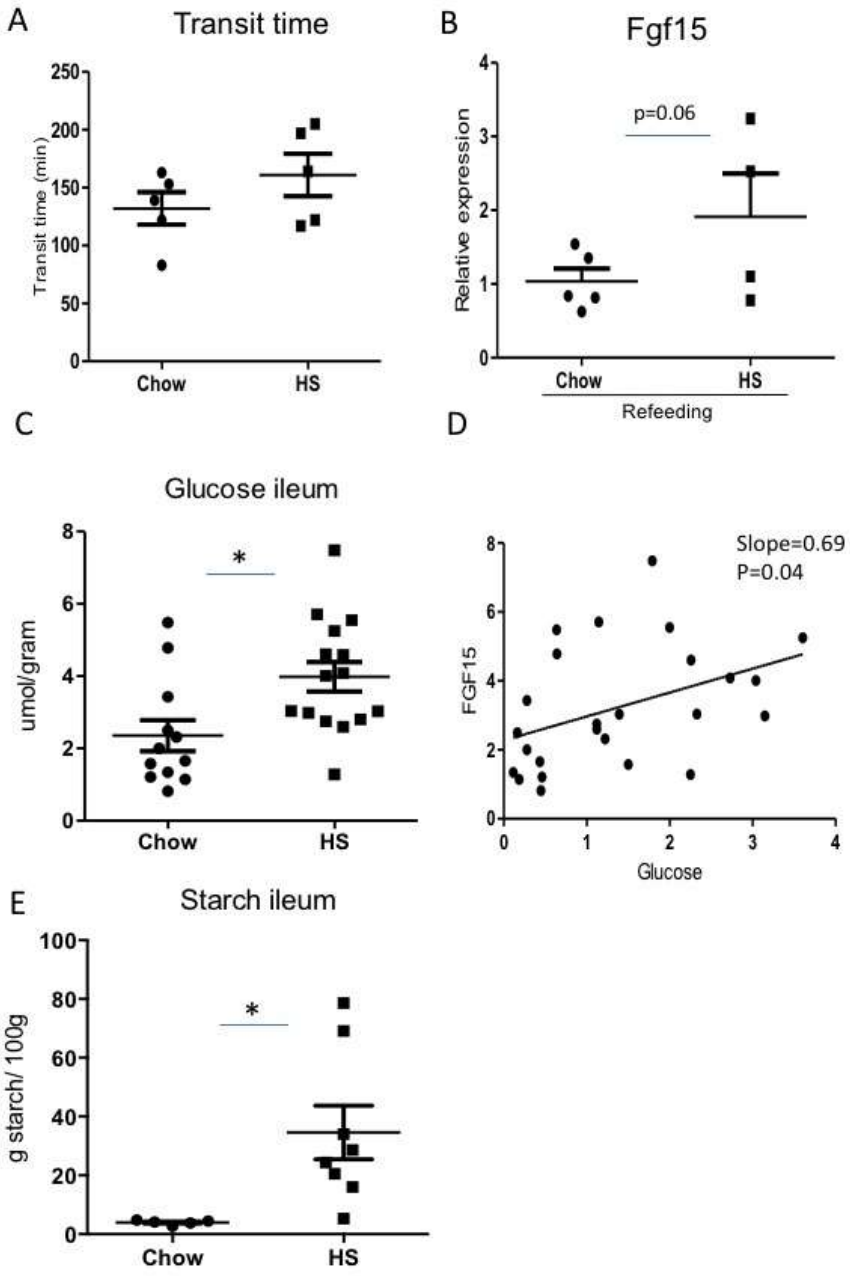

Figure 3.5 The HS diet has a direct effect on Fgf15 expression, most likely due to ileal exposure to starch and glucose. Transit time was tested using Evans Blue mixed with the corresponding diets in mice that had been fed the chow or HS diet for three months (A). To test the direct effects of the HS diet, mice on a chow background were fasted for 12 hours overnight before being refed for 3 hours with either chow or the HS diet (B). In mice fed the chow or HS diet for 3 days to 2 weeks, glucose was determined in the luminal content of the ileum, showing to be increased in the HS group and correlating with *Fgf15* expression (C). *Significance was tested using an unpaired t-test between chow and HS diet (*= p<0.05), correlation was tested using Pearson correlation n≤4 for all groups.*

3.3.5 Inhibiting starch digestion by acarbose treatment

To determine the causality of starch and starch digestion in the observed increase in ileal *Fgf15* expression, mice were treated with acarbose in addition to the HS diet. Acarbose is an alpha-glucosidase inhibitor that is commonly used in the management of hyperglycaemia. It is a noninsulinotropic oral antidiabetic agent that competitively blocks alpha-glucosidases and thereby inhibits the digestion of di, oligo- and polysaccharides. α-Glucosidases are enzymes located in the brush border membrane of the small intestine that cleave di- and oligosaccharides into monosaccharides to enable their uptake into the enterocytes. Acarbose is a pseudotetrasaccharide and similar to that of natural oligosaccharides, however it has a 10^4 to 10^5 times higher affinity for α-glucosidases and can therefore decrease the number of available enzymes for the digestion of starch-derived oligosaccharides (Rosak and Mertes 2012). Thus, under acarbose treatment, starch digestion is decreased which leads to less glucose uptake into epithelial cells.

In this experiment, mice were treated with acarbose at a dose of 50mg per kg of body weight per day for 2 weeks, and this was administered via the drinking water (figure 3.6A). To confirm the decreased uptake of starch in the small intestine, starch was quantified in the caecum showing an increase in the acarbose treated mice (figure 3.6B). Subsequently, *Fgf15* expression was quantified in the ileum and showed a dramatic decrease in the acarbose-treated mice (figure 3.6C), which correlated with an increase in Cyp7a1 expression in the liver (figure 3.6D). Additionally, a trend towards decreased serum cholesterol levels in acarbose-treated mice was seen (figure 3.6E). This effect is in line with the previous observation that *Fgf15* and *Cyp7a1* expression have effects on cholesterol levels. Taken together, these data indicate that the conversion of starch to glucose is causal in the upregulation of the signalling molecule *Fgf15* in the ileum.

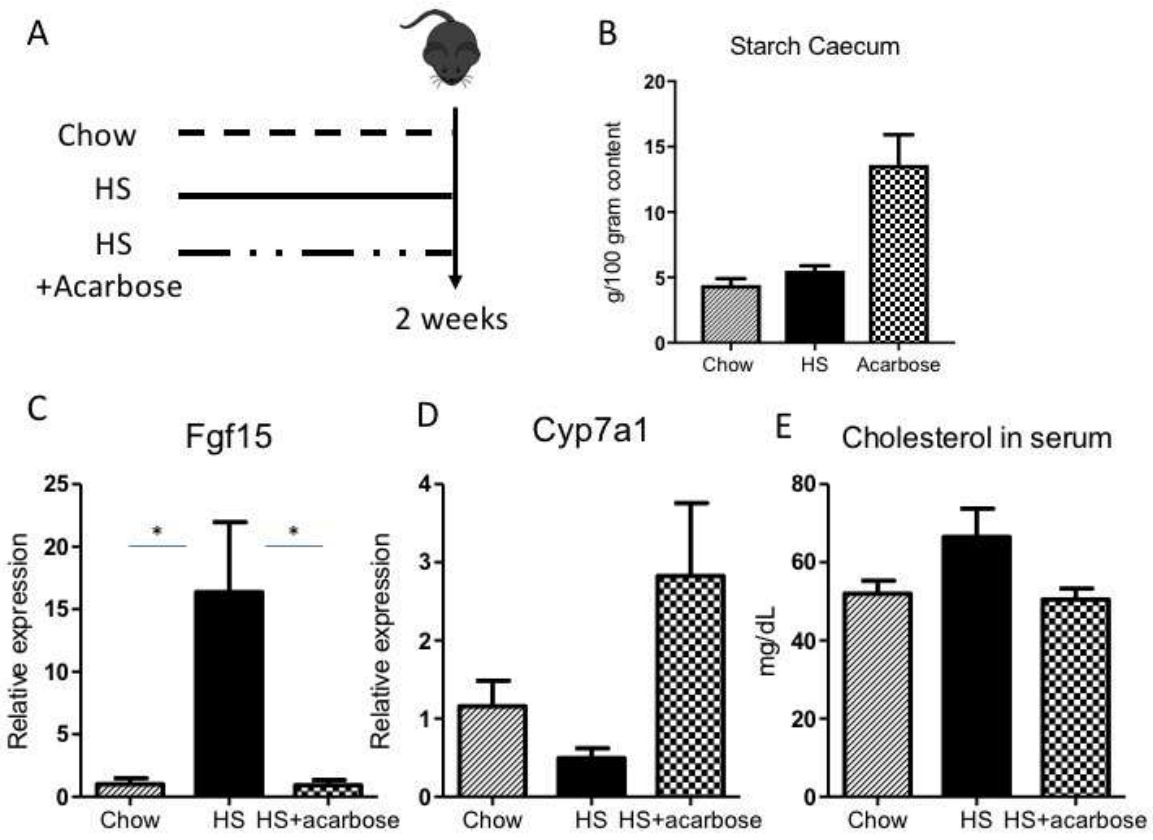

Figure 3.6 Inhibiting starch digestion by acarbose decreases *Fgf15* expression and consequently increases hepatic *Cyp7a1* expression. To inhibit starch digestion one group of mice was treated with acarbose (50mg / kg BW) for two weeks (A), shown to be effective by an increase in starch found in the caecum thus escaping digestion the in small intestine (B). *Fgf15* expression in the ileum is downregulated by acarbose treatment (C) which correlated with an upregulation of hepatic *Cyp7a1* expression (D) and a trend towards lower serum cholesterol levels (E). *Significance was tested using 1-way ANOVA with Bonferroni post-hoc test between chow and HS diet (*= p<0.05) n≤5 for all groups*

3.3.6 Intracellular Glucose in the epithelial cells after uptake from the intestinal lumen increases Fgf15 expression

Since starch digestion into glucose was determined to be essential for the starch-induced increase in *Fgf15* expression, the direct effects of glucose were investigated. Although glucose is normally absorbed in the duodenum and jejunum, overloading the mouse small intestine with glucose has been shown to induce glucose uptake in the ileum (Bode et al. 1981). Therefore, mice on a chow diet were treated with a one-time dose of glucose (2 gram / kg BW, a concentration often used in glucose tolerance tests (Andrikopoulos et al. 2008)) via oral gavage and the response in ileal *Fgf15* expression was tested over time (Appendix 3A). Blood glucose levels were measured 15, 30, 60, 90 and 120 minutes after OG and showed an expected initial increase and consequent normalization (Appendix 3B). Two mice per time point were sacrificed 30, 60, 90 and 120 minutes after OG to measure intestinal and hepatic gene expression. *Fgf15* expression showed a time-dependent increase after glucose OG with a peak at 90 minutes (Appendix 3C), and this timepoint was selected for further experiments. Additionally, hepatic *Cyp7a1* decreased over time (Appendix 3D) and, as previously described, hepatic glucose metabolism genes responsive to *Fgf15* (Potthoff et al. 2011) show a decrease at the same time that *Fgf15* expression increases (Appendix 3E).

To gain sufficient significance in the increase of *Fgf15* after glucose OG the experiment was repeated with 5 mice per group both before and 90 minutes after OG. Additionally, a third group of mice received the same dose of glucose (2g/ kg BW) via intraperitoneal (IP) injection to test if glucose needed to reach the ileum via the intestinal lumen in order to increase *Fgf15* expression (figure 3.7A). In line with the preliminary results, *Fgf15* expression was found to be significantly higher 90 minutes after glucose OG (figure 3.7B). Mice who received the glucose load via IP injection, circumventing the intestinal lumen, did not show a similar increase (figure 3.7B). As a proof of concept, glucose levels in the intestinal lumen of the ileum were measured and show an increase in OG treated mice (figure 3.7C). Hepatic *Cyp7a1* expression was decreased in both OG and IP mice (figure 3.7D), which might be explained by an increase in hepatic FXR activity in response to increased blood glucose levels (Berrabah et al. 2014).

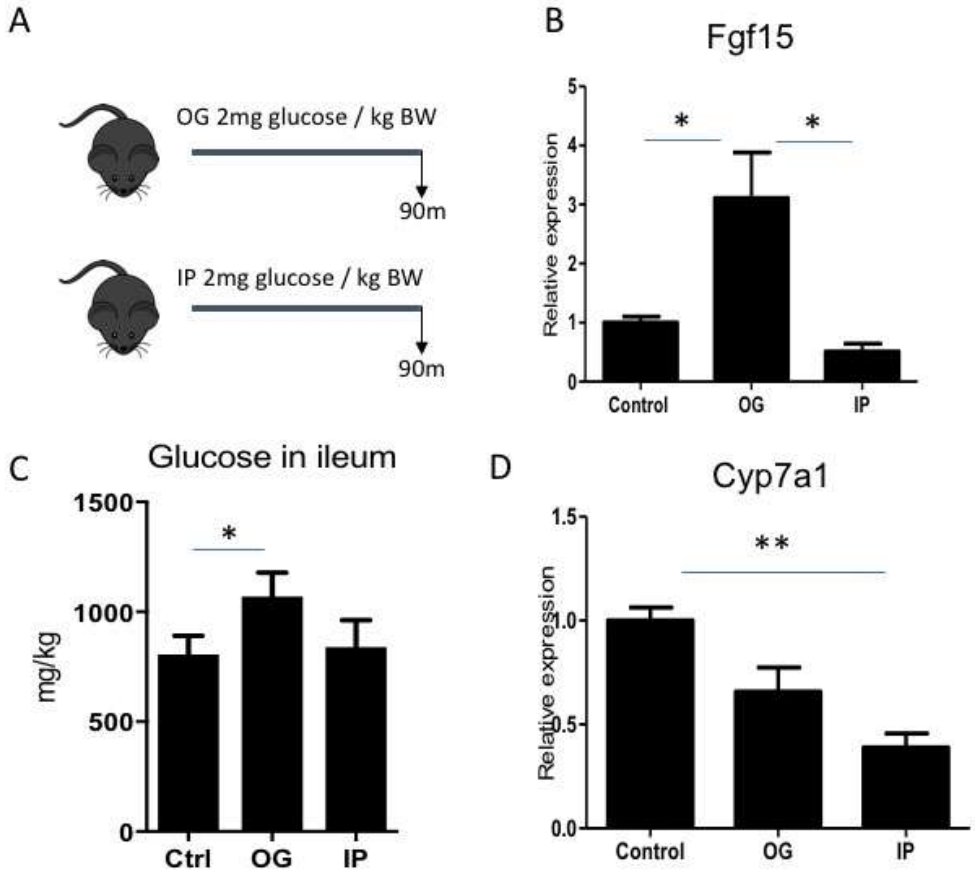

Figure 3.7 Glucose reaching the ileal enterocytes via the apical membrane induces *Fgf15* expression. Mice were treated either by oral gavage (OG) or intraperitoneal injection (IP) and sacrificed before or 90 minutes after treatment (A). *Fgf15* expression in the ileum was significantly higher after OG but not IP (B). Glucose from OG treated mice reaches the ileum (C). Hepatic *Cyp7a1* expression is downregulated in both OG and IP treated mice (D). *Significance was tested using 1-way ANOVA with Bonferroni post-hoc test between chow and HS diet (*= p<0.05) n≤5 for all groups*

3.3.8 Bile acids are essential for *Fgf15* expression, independent of diet

As mentioned previously, bile acid profiles in the ileum were not found to be consistently different between chow and HS fed mice (figure 3.1E and table 3.1). Therefore, we designed an experiment to investigate the requirement of bile acids for the observed difference in *Fgf15* expression. Hence, mice were treated with the bile acid sequestrant cholestyramine twice daily via oral gavage. Cholestyramine is a positively charged non-

digestible resin that binds to bile acids in the intestine to form insoluble complexes that leave the body via the faeces (Scaldaferri et al. 2013). Thus, cholestyramine inhibits the reabsorption of bile acids in the ileum. Mice on both the chow and the HS diet were treated with cholestyramine and *Fgf15* expression was found to be abolished on both diets (figure 3.8B). This indicates that bile acids are essential for the expression of *Fgf15* in the ileum, independent of the starch content of the diet.

3.3.9 The farnesoid X receptor is involved in the increase of Fgf15 by starch and glucose

The most well-known regulation of *Fgf15* is via the transcription factor FXR, which can be activated by bile acids (Dawson and Karpen 2015). Since bile acids were found to be essential in the regulation of Fgf15 in mice on both the chow and the HS diet, the role of FXR was determined further. To investigate the role of FXR in the regulation of *Fgf15* a natural FXR antagonist, guggulsterone, was used. Guggulsterone is a steroid that can be found in the guggul plant and has been used in Ayurvedic medicine since 600 BC to treat a wide variety of diseases including lipid disorders. Mice on the chow and HS diet were treated with 20 mg/kg BW via daily oral gavage and showed a decrease in ileal *Fgf15* expression for both diets (figure 3.8C). Another ileal FXR target gene, *Fabp6*, showed a similar decrease under guggulsterone treatment indicating that guggulsterone inhibited FXR activity in our experiment (figure 3.8D).

To investigate the posttranscriptional effects of glucose on FXR protein expression, western blots were performed on protein extracts of ileal tissue at time-points after glucose oral gavage showing an increase in FXR protein over time (figure 3.8E), while no increase was observed at gene expression level (data not shown). Other ileal FXR target genes, *Shp* and *Fabp6*, were quantified after glucose treatment by OG and IP, showing a significant increase in OG treated mice only (figure 3.8F). Taken together, these data indicate a role for the transcription factor FXR in the upregulation of *Fgf15* expression by starch and glucose.

In combination with the cholestyramine results this confirms that glucose, bile acids and FXR are needed to achieve the observed increase in *Fgf15* expression, leading us to speculate that FXR undergoes glucose-hexosamine-derived O-linked B-N-acetylglucosamine modification (GlcNAcylation). O-GlcNAcylation leads to an increase

of FXR protein stability, transcriptional activity, and chromatin binding through SMRT inactivation, which afterwards would lead to increased target gene expression after activation by bile acids (Benhamed et al. 2014).

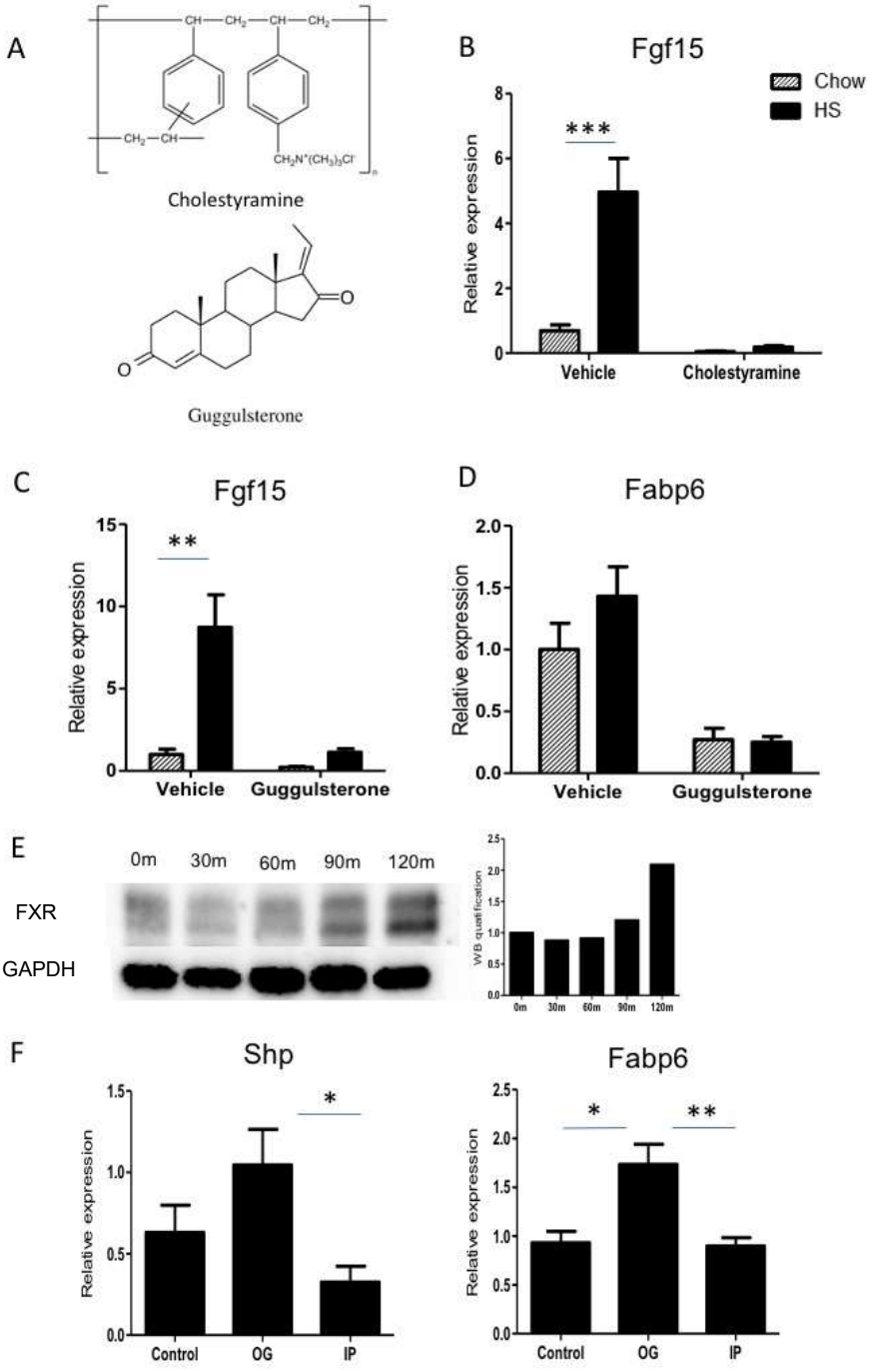

Figure 3. 8 Both bile acids and the transcription factor FXR are involved in the upregulation of *Fgf15* by starch and glucose. Mice were treated with cholestyramine (A), a bile acid sequestrant, which abolishes *Fgf15* expression in both chow and HS fed mice (B). To investigate the role of FXR mice were treated with the natural FXR antagonist guggulsterone (A), and *Fgf15* expression was downregulated under both dietary conditions (C). Additionally, *Fabp6*, another ileal FXR target gene was also downregulated by guggulsterone (D), indicating that it successfully depressed FXR activity. FXR protein was quantified in response to an OG glucose load and showed upregulation over time (E). Furthermore, the ileal FXR target genes *Shp* and *Fabp6* were upregulated by an oral glucose load but not if the glucose was administrated via IP injection (F). *Significance was tested using 2-way ANOVA (B, C, D) or 1-way ANOVA (F) with Bonferroni post-hoc test between chow and HS diet (***= p<0.001, **= p<0.01, *= p<0.05).*

3.3.11 Long term effects of increased FGF15 expression on circulating bile acids

As shown before, the starch induced increase in *Fgf15* leads to an increase of cholesterol levels, however also other long-term health effects were observed. Firstly, we investigated the effects of the changes of both ileal *Fgf15* and hepatic *Cyp7a1* expression on circulating bile acids. Serum bile acids were determined via HPLC-MS and large differences were found between the diet groups after 6 months of feeding (figure 3.9A). Notably, these differences were not observed at the shorter time points (3 days – 2 weeks, data not shown). This disturbance in circulating bile acids could potentially have wide-spread health effects by these bile acids activating the ubiquitously expressed bile acid sensing transcription factor TGR5 (Kawamata et al. 2003).

3.3.12 Long-term glucose induction of FGF15 potentially leads to hepatic carcinoma development

Furthermore, in our long-term experiment (6 months), we encountered hepatic carcinoma in 2 out of 9 HS fed mice (figure 3.9B) while no carcinoma was encountered in the chow fed mice (0/10). Induction of FGF15/19 has previously been linked to the development of hepatic carcinoma (Uriarte et al. 2015; Uriarte et al. 2013); here we show that glucose-dependent induction of *Fgf15* can contribute to the development of liver cancer in mice. Additionally, the alanine aminotransferase (ALT) was found to be higher in the serum of mice exposed to the HS diet for 6 months (figure 3.9C), indicating liver damage. At the shorter time points (3 days and 2 weeks) no significant differences in transaminases were

observed (data not shown), however a trend toward more cell division, as measured by *Ki67*, Cyclin D (gene *Ccdn1*) and Survivin (bene *Birc5*) gene expression is already observed at these time points (figure 3.9D). Further, gene expression in these cell proliferation genes was found to be significantly upregulated after 6 months of feeding even though the mice with hepatic carcinoma were excluded from the gene expression data set (figure 3.9D). Taken together, this indicates that the glucose induction of *Fgf15* is substantial enough to contribute to the development of hepatic carcinoma in mice.

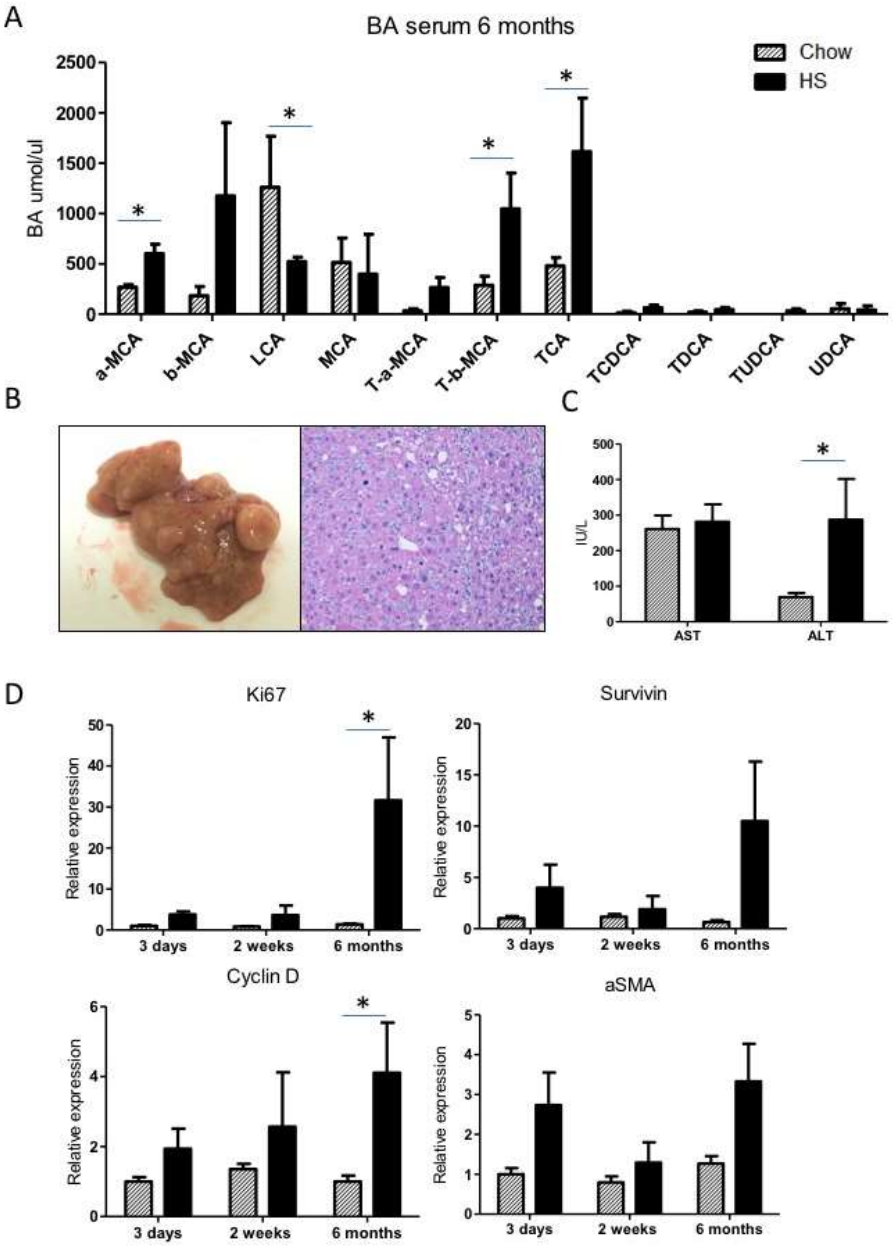

Figure 3.9 Long term overexpression of *Fgf15* leads to alterations in circulating bile acids and increases the chances of developing hepatocarcinoma in mice. After 6 months of feeding chow versus HS diet large differences in circulating bile acids are observed (A). Furthermore, hepatic carcinoma was encountered in 2/9 HS fed mice (B), compared to 0/10 chow fed mice, and serum ALT levels were found to be increased in HS fed mice (C). Trends towards more cell proliferation and collagen production in mice fed the HS diet can be found at early time points (3 days – 2 weeks, D) and these differences become significant after 6 months even though the mice with profound liver cancer are excluded from gene expression data set (D). *Significance was tested using 2-way ANOVA with Bonferroni post-hoc test between chow and HS diet (*= $p<0.05$) $n\leq5$ for all groups*

3.4 Discussion

Here, for the first time, we show that dietary starch can increase the expression of the important signalling molecule FGF15/19 in the ileum. A HS diet can lead to an increase in glucose absorption in ileal enterocytes which in turn provokes an increased expression of FGF15/19, possibly via GlcNAcylation of the bile-sensing transcription factor FXR. Since FGF15/19 is involved in blood glucose homeostasis by activating relevant pathways in the liver (Kir et al. 2011; Wu et al. 2010; Potthoff et al. 2011), this speculatively may be a response of the body to an overload of starch.

Although we are, to the best of our knowledge, the first to highlight this effect, indications have surfaced in previous scientific papers. Most research groups compare the HS diet to a purified HF diet, as was also used in the WUR studies. De Wit *et al* (De Wit et al. 2008) previously identified a difference in *Fgf15* expression (by performing microarray analysis of the ileum) in mice on the HS and HF diet, but assumed a downregulation of *Fgf15* by dietary fat. Here we show that rather than the dietary fat decreasing *Fgf15* expression, a surplus of dietary starch is increasing its expression. The biological activities surrounding the increase in *Fgf15* are shown by its effect on both *Cyp7a1*, the rate limiting enzyme in *de novo* bile acid synthesis, and on blood and liver cholesterol levels. In this regard, regulation of FGF15/19 expression has shown to affect cholesterol levels in both mouse models (Stroeve et al. 2010) and human intervention studies (Harrison et al. 2018).

The combination of scientific literature showing a reduction in *Fgf15* expression both after antibiotic treatment (Miyata et al. 2009) and in germ free mice (Sayin et al. 2013b), and the major differences in the small intestinal microbiota we identified led us to a hypothesized role for the microbiota in the regulation of *Fgf15* expression. Conversely, antibiotic-treated mice on the HS diet, in which the small intestinal microbiota was successfully abolished, showed no decrease in *Fgf15* but a slightly greater increase in *Fgf15* expression compared to conventionally raised HS-fed mice. Since we determined starch to be responsible for the *Fgf15* alterations, this difference could potentially be due to the quantity of available starch present in the ileum, as it has been shown that the microbiota can ferment digestible starch (McBurney, Cuff, and Thompson 1990). So, potentially the small intestinal microbiota negatively influences the amount of starch reaching the ileal enterocytes and depleting the microbiota leads to an increase in ileal

starch exposure. Of note, our results are in line with previous literature, as in chow fed mice we observed a decrease in *Fgf15* expression.

Acarbose is an antidiabetic drug that inhibits the hydrolysis of complex carbohydrates and thereby reduces glucose uptake in the small intestine. Acarbose treatment, therefore, was used to show the role of starch digestion in *Fgf15* gene expression regulation. Recently, the effects of acarbose on the microbiota and bile acid profiles in humans have been studied showing an increase in circulating unconjugated bile acids. As a result of the bile acid profiles, an increase in FGF19 would be expected, however a significant decrease in circulating FGF19 was observed (Gu et al. 2017). This, in line with our findings and proposed mechanism, could be explained by the decreased digestion of starch and other oligosaccharides in the ileum. Additionally, a slight decrease in blood cholesterol levels was observed in the acarbose treated participants (Gu et al. 2017), similar to the effects we observe in our mouse model. Further emphasizing the human relevance of the here elucidated mechanism of FGF15/19 upregulation by glucose is given in a postprandial study (Morton et al. 2014). Here, participants were given a fat, protein or carbohydrate drink in a cross-over design and their response in circulating FGF19 was measured. The carbohydrate drink consisted of mostly glucose and increased circulating FGF19 levels in blood significantly compared to both the fat and protein drink, which is consistent with the here observed increase of *Fgf15* expression after glucose OG in mice. Together with previously discussed human acarbose study (Gu et al. 2017), this indicates that the upregulation of *Fgf15* expression after ileal exposure to starch and/or glucose is most likely also relevant for humans.

To further elucidate the molecular mechanism behind the regulation of FGF15/19, the involvement of both bile acids and FXR were identified as essential in the increase of *Fgf15* expression by glucose. This would be in line with a study performed at Groningen University by Herrema *et al* (unpublished), where they fed wild type (WT) and intestinal specific FXR knockout mice a high dextrose diet (consisting of mostly glucose) or chow diet and observed an increase in Fgf15 expression in the ileum only in WT mice fed the dextrose diet. Additionally, they observed an even higher increase in WT mice fed the dextrose diet in the morning compared to the evening which could be explained by mice

consuming most of their food during the night time (Vanderlip 2001); hence more glucose being present in the ileum in the morning. For all murine studies conducted for this thesis, sacrifices took place in the morning.

Although not much is known about ileal FXR regulation by glucose, glucose has been shown to increase hepatic FXR in a dose-dependent manner in rats on both gene and protein expression and activity (Duran-Sandoval et al. 2004). Glucose is capable of altering FXR activity via glucose-hexosamine-derived O-linked B-N-acetylglucosamine modification (GlcNAcylation). O-GlcNAcylation leads to an increase of FXR protein stability, transcriptional activity, and chromatin binding through SMRT inactivation (Benhamed et al. 2014). This has shown to regulate hepatic FXR in vivo during fasting and refeeding cycles (Berrabah et al. 2014). So, higher glucose levels can stabilize FXR protein via GlcNAcylation, however activation of FXR is still needed to show this increased activity. This is in line with our results where we need both FXR and its natural activator, bile acids, to induce the increased expression of *Fgf15* in starch fed mice (figure 3.12).

Surprisingly, in our model *Fgf15* is the most responsive FXR target gene after the possible stabilization of FXR protein. Only after glucose OG we were able to show other FXR target genes to be upregulated in the ileum (figure 3.9F). We hypothesize that *Fgf15* expression is particularly sensitive to alteration in FXR stability, however more research will be required to reveal the molecular mechanism behind this.

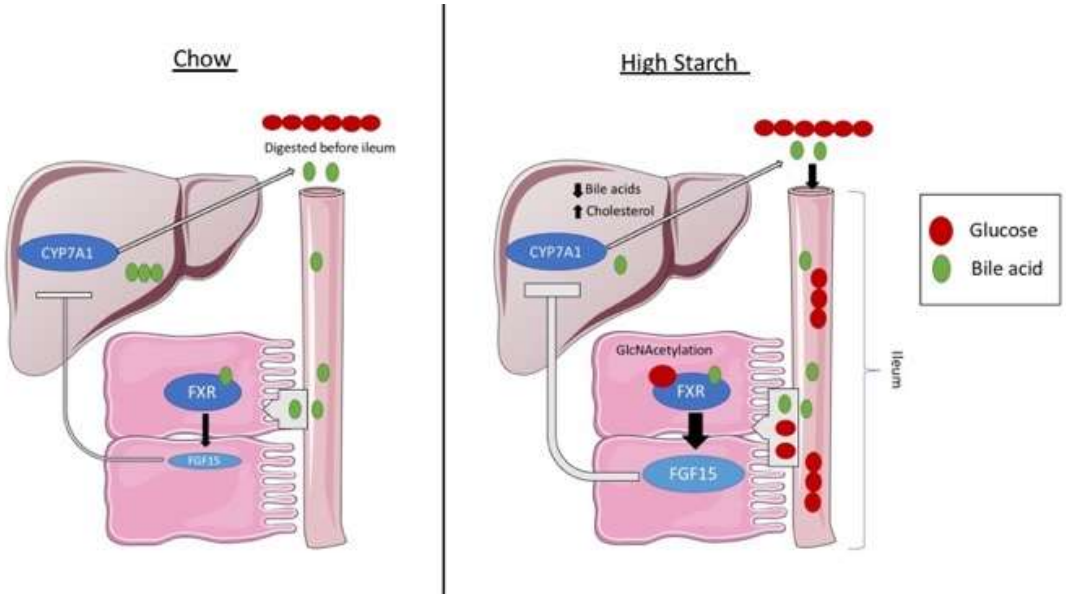

Figure 3.10 Hypothesis on the molecular mechanism by which starch increases the expression of FGF15/19. In addition to bile acid reabsorption taking place in the ileum, under high starch conditions glucose is also absorbed into ileal enterocytes. Increased intracellular glucose levels can lead to GlcNAcylation of FXR, stabilizing the protein. Bile acids activate FXR in both the control and the high starch conditions, but due to the increase in protein stabilization in the high starch fed group, there is more production of the important signalling molecule FGF15/19 in the high starch fed group. This consequently leads to more inhibition of CYP7A1, the rate limiting enzyme in de novo bile acid synthesis, and therefor to less bile acid production and a rise in cholesterol levels, among other health effects. *This figure was produced using and adapting figures from* ("SMART - Servier Medical ART" 2018).

Long term overexpression of FGF15/19 has a number of detrimental health effects; one being higher blood cholesterol levels due to a reduced conversion of cholesterol into bile acids by hepatic CYP7A1 (S. A. Harrison et al. 2018; Dawson 2015). Here, we show dietary regulation of Fgf15 by starch can increase cholesterol levels, and most likely by the same mechanism. Furthermore, serum bile acid profiles were significantly altered after 6 months of feeding, which can influence other organs via the bile acid sensing G-coupled protein receptor TGR5. TGR5 is expressed ubiquitously in both mice and humans, and its expression has been shown in a wide distribution of organs including endocrine glands, adipocytes, muscles, intestine, spleen and lymph nodes (Kawamata et al. 2003). LCA is the most potent agonist of TGR5 (Duboc, Taché, and Hofmann 2014), and is downregulated in mice fed the HS diet for 6 months, however a slightly less potent agonist,

TCA, is upregulated under these conditions. Further research is needed to identify the effects of LCA and TCA in modifying bile acid profiles on TGR5 activation in other organs and its health effects.

FGF15/19 has shown to be hepatoprotective during acute liver injury; while it is pro-carcinogenic if chronically activated. Consequently, interfering with the FGF19 – FGFR4/KLB signalling pathway in liver cancer is regarded a new strategy in fighting this disease (Alvarez-Sola et al. 2017; Miura et al. 2012; Hyeon et al. 2013; Hagel et al. 2015). Additionally, *Fgf15* KO mice develop less hepatocellular carcinoma and fibrosis under the treatment of diethylnitrosamine compared to their wildtype littermates. *Fgf15* KO mice show less cell proliferation in the liver, as shown by Ki-67 and less fibrosis as shown by aSMA (Uriarte et al. 2015). Furthermore, *Fgf15* has previously been shown to induce cyclin D expression in a partial hepatectomy mouse model (Li et al. 2018). In the current feeding studies, chronic overexpression of *Fgf15* is induced in the HS mice and after 6 months hepatic carcinoma was observed in 2 out of 9 HS fed mice while no hepatic carcinoma was observed in the chow fed animals. After only 3 days and 2 weeks of feeding of the HS diet, a small trend towards more cell proliferation (shown by *Ki-67* gene expression) and fibrosis, (shown by aSMA expression) could already be observed. This correlates with the higher levels of *Fgf15* in these animals. Taken together this emphasizes the severity of the chronic diet-induced induction of *Fgf15*.

In conclusion, we demonstrate that ileal exposure to glucose, either directly or in the form of starch, increases the expression of the important signalling molecule *Fgf15*, independent of the microbiota and most likely via GlcNAcylation of ileal FXR.

Chapter 4 Soluble fibres suppress the production of signalling molecule Fgf15 in the ileum most likely independent of the microbiota

Thesis Britt Blokker

Name study	Where	Performed by candidate?	Samples available
FOS study	UEA	Yes	All
FOS + antibiotics study	UEA	Yes	All
Soluble fibre study	UEA	Yes	All

As briefly discussed in the last chapter, starch content is not the only difference between the chow and HS diet. Another important factor is the lack of (soluble) fibres in the HS diet. Therefore, in this chapter, the role of dietary soluble fibres on (the gut-liver axis and in particular on) *Fgf15* expression will be discussed.

4.1 Introduction

Fibres are non-digestible carbohydrates that can be found in mainly plant-based foods and can be divided into two broad groups; insoluble and soluble fibres. Insoluble fibres are, as the name suggests, not soluble in water and are poorly fermentable by the gut microbiome. Soluble fibres are soluble in water and are fermented by bacteria in our GI tract, leading to the production of short chain fatty acids (SCFAs) (Dhingra et al. 2012; Slavin 2013). Epidemiological studies have found an inverse correlation between dietary fibre intake and blood cholesterol levels (Brown et al. 1999) and the risk of cardiovascular disease (Threapleton et al. 2013). The cholesterol lowering effect of soluble fibres has also been shown in multiple animal models, e.g. mice (S. Han et al. 2015a) and pigs (P. Gunness et al. 2016).

While some hypotheses exist, to this day it is not completely understood how soluble fibre can decrease blood cholesterol levels. One of the proposed mechanisms is via modulation of bile acid metabolism, as this is a major determinant of cholesterol metabolism (Purnima Gunness and Gidley 2010). As discussed in detail in the introduction of this thesis, bile acids are produced from cholesterol in the liver, and bile acid that escape reabsorption in the ileum are an important pathway of cholesterol excretion in mammals (Kuipers, Bloks, and Groen 2014). Additionally, dietary fibres are known to have a large influence on the gut microbiota and the production of SCFAs in both rodents and humans (David et al.,

2014; Lange et al., 2015). A change in the microbiota could infleunce bile acid metabolism via the enzyme BSH which is uniquely produced by bacteria and leads to deconjugation of bile acids and eventually the formation of secondary bile acids (Ridlon et al. 2014; Jones et al. 2008). Changes in the microbiota and deconjugation of bile acids has previously been linked to changes in the expression of *Fgf15* (Li et al., 2013; Sayin et al., 2013)

Combining the cholesterol-lowering and microbiota altering effects of dietary fibres with the known influence of the microbiota on *Fgf15* expression led us to our research question; how do dietary fibres regulate *Fgf15* expression in the small intestine and how does this effect cholesterol levels? Here we show that bile acid metabolism is involved in the cholesterol-lowering effects of soluble fibres via regulation of *Fgf15* expression in the ileum which leads to a decrease in bile acid production in the liver. Interestingly, we demonstrate that this effect is most likely independent of the microbiota.

4.2 Materials and methods

4.2.1 Murine studies

C56BL6/J mice bred in the DMU of the UEA were used for all murine studies (except WUR study, see below), starting at the age of 10-12 weeks, with a group size of minimum 5 and maximum 10 mice. At the end of the experiments mice were sedated with a mixture of 4% isoflurane (IsoFlo®, Abbott, ND), nitrous oxide (70%) and oxygen (30%) and blood samples were collected via cardiac puncture. The small intestine was collected and arranged in three equal parts, of which the last part was identified as the ileum. Additionally, the liver was collected. All samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis or fixed in 10% formalin (include if you show histology).

All experimental procedures and protocols used in these studies were reviewed and approved by the Animal Welfare and Ethical Review Body and were conducted according to the specifications of the United Kingdom Animals Scientific Procedures Act, 1986.

4.2.2 Diets

Mice were fed either a standard chow diet (RM3-P, Special Diets Services, UK), a HS purified diet (D12450H, Research Diets, USA) for the indicated duration per experiment (table 4.1).

Table 4.1 Dietary composition per kilogram of diet. The high starch (HS) diet contains more starch and sucrose and less fibre compared to the chow diet. In the purified diet (HS and high fat (HF) from Research Diets) starch is exchanged for fat.

	Chow diet SDS diets RM3	HS diet D12450H
Energy content	3.35 kcal/g	3.82 kcal/g
Protein	27.28 En%	20 En%
Carbohydrate	61.24 En%	70 En%
Fat	11.48 En%	10 En%
Corn Starch (g)	338.8	452.2
Maltodextrin (g)	0	75
Sucrose (g)	43.7	172.8
Dietary fibre (g)	161.5 Mix of soluble and insoluble	50 Cellulose only
Vitamins See appendix 1	Unknown mix	1 g V10001C
Minerals See appendix 1	Unknown mix	50 g S10026B

4.2.3 Intervention studies

FOS study

In addition to the chow and HS diet, mice received the soluble fibre fructo-oligosaccharides (FOS, Sigma-Aldrich, F8052) via the drinking water for the duration of one week. FOS was added to the drinking at a concentration of 30g/L to reach a daily intake of approximately 120mg per mouse.

FOS and antibiotics study

Mice on a chow background were switched to a clean cage and to the HS diet for 1 week with added FOS in the drinking water at a concentration of 30g/L. During this week mice were oral gavaged once daily with a cocktail of vancomycin (50mg/kg BW, Sigma-Aldrich, 94747), Neomycin (100mg/kg BW, Sigma-Aldrich, N6386), Metronidazol (100mg/kg BW, Sigma-Aldrich, M1547) in 0.9% saline. Additionally, mice received ampicillin (1g/L, Sigma-Aldrich, A0166) via drinking water. Mice were transferred to a clean cage every other day for the duration of this experiment. Similar antibiotics protocol used in (Zarrinpar et al. 2018).

Soluble fibre studies

In collaboration with Research Diets Inc (New Brunswick, USA), new diets were designed. Firstly, a new HS diet was constructed containing less sucrose compared to the original HS diet. Secondly, diets containing soluble fibre were also made, as no soluble fibres were present in the semi-purified HS diets. Thirdly, three different soluble fibre diets were designed, containing either 75 gram of pectin, inulin or fibersol. Calorie intake was normalized to the HS diet by slightly decreasing the starch content. For the full contents of these diets please see table 4.2.

Mice were fed these diets for a duration of two weeks, and additionally a group of mice fed the chow diet was added to this experiment.

Table 4.2 Dietary composition of the added soluble fibres diets. Rodent diets with 10 En% fat with 50g Cellulose and added with 75g inulin, pectin or soluble corn fibre (Fibersol).

Product #	**D12450J**		**D18012101**		**D18012102**		**D18012103**	
	10 kcal% Fat		*Modified w/*		*Modified w/*		*Modified w/*	
	50 g Cellulose		*50 Cell/75 Inulin*		*50 Cell/75 Pectin*		*50 Cell/75 Fibersol-2*	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	19	*20*	18	*20*	18	*20*	18	*20*
Carbohydrate	67	*70*	62	*70*	62	*70*	62	*70*
Fat	4	*10*	4	*10*	4	*10*	4	*10*
Total		*100*		*100*		*100*		*100*
kcal/gm	3.85		3.68		3.68		3.69	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*	3	*12*	3	*12*
Corn Starch	506.2	*2024.8*	478	*1912*	478	*1912*	476.2	*1905*
Maltodextrin 10	125	*500*	125	*500*	125	*500*	125	*500*
Sucrose	68.8	*275*	68.8	*275*	68.8	*275*	68.8	*275*
Cellulose, BW200	50	*0*	50	*0*	50	*0*	50	*0*
Inulin, Orafti HP	0	*0*	75	*113*	0	*0*	0	*0*
Pectin, Tic Gums 1400	0	*0*	0	*0*	75	*113*	0	*0*
Fibersol-2 (Resistant Maltodextrin)	0	*0*	0	*0*	0	*0*	75	*120*
Lard	20	*180*	20	*180*	20	*180*	20	*180*
Soybean Oil	25	*225*	25	*225*	25	*225*	25	*225*
Mineral Mix S10026	10	*0*	10	*0*	10	*0*	10	*0*
Dicalcium Phosphate	13	*0*	13	*0*	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*
Yellow Dye #5, FD&C	0.04	*0*	0.025	*0*	0	*0*	0	*0*
Red Dye #40, FD&C	0	*0*	0.025	*0*	0	*0*	0.025	*0*
Blue Dye #1, FD&C	0.01	*0*	0	*0*	0.05	*0*	0.025	*0*
Total	**1055.05**	***4057***	**1101.85**	***4057***	**1101.85**	***4057***	**1100.05**	***4057***
gm								
Total Fiber	50.0		125.0		125.0		125.0	
Insoluble Fiber	50.0		50.0		50.0		50.0	
Soluble Fiber	0.0		75.0		75.0		75.0	
gm%								
Total Fiber	4.7		11.3		11.3		11.4	
Insoluble Fiber	4.7		4.5		4.5		4.5	
Soluble Fiber	0.0		6.8		6.8		6.8	
Fiber contents listed above are estimates based on these fiber sources containing 100% fiber and either 100% as soluble or insolu								
Assayed levels: Cellulose = 93%, Inulin = 96%, Pectin = 93%. Fibersol-2 = 90% (manufacturer data)								
Inulin contains 1.5 kcal/gm as per Roberfroid, 1999 (J. Nutr. 129: 1436S–1437S, 1999)								
Pectin doesn't have a specified kcal/g value, so have chosen 1.5 kcal/g to match inulin. MAP								
Fibersol-2 has 1.6 kcal/g according to information from ADM/Matsutani								

4.2.3 Gene expression

RNA of liver and intestinal scraping samples was isolated. Samples were homogenised in 1 ml Trizol (Qiazol, Qiagen, UK) for 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France). After homogenization samples were transferred to a new Eppendorf tube and 200 µl Chloroform was added. Samples were centrifuged for 15 minutes at 12.000 RCF 4˚C and the clear layer was transferred to a new collection tube. Iso-propanol was added and after mixing, resting for 5-10 minutes and centrifugation for

10 minutes at 12.000 RCF 4˚C an RNA pellet was left. The pellet was washed in 85% ethanol and dissolved in 100 µl RNase Free water. RNA concentration was measured with a Nanodrop (Thermo Scientific, Wilmington, USA). cDNA was synthesized from 2 µg of RNA which was first treated with DNase (DNase kit, Invitrogen, 18068-015) and subsequently reverse transcription was performed. A quantitative polymerase chain reaction (Q-PCR) was performed using SYBR green master mix (Applied Biosystems, Thermo Fisher Scientific, UK) according to the producer's instructions. Reactions were performed using 384 wells (Applied Biosystems, Thermo Fisher Scientific, UK) using 5µL of SYBR green and primer mix (for a list of the primers used, see table 4.3) and 1.5µL of cDNA. The reaction was initialized at 50°C for 5 minutes and 95°C for 2 minutes, after which 40 cycles of denaturation (95°C 15 seconds) and annealing/extension (59°C 1 min) were performed. Afterwards, a melting curve was created and checked for a single product per gene. ΔCT values were calculated by subtracting the CT value of the housekeeping gene TBP from the gene CT value. ΔΔCT was calculated relative to control samples (Livak and Schmittgen 2001).

Table 4.3 Primers used for q-PCR measurements in small intestine and liver.

Gene	Forward	Reverse
TBP (hk)	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT
GAPDH (hk)	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
FGF15	CAGTCTTCCTCCGAGTAGCG	TGAAGACGATTGCCATCAAG
CYP7A1	GAGCGCTGTCTGGGTCACGG	GCCAGCCTTTCCCGGGCTTT

4.2.4 Bile acid measurements

As no bile acid measurements were performed in house at the time of arrival, we set up our own method in collaboration with the metabolomics unit of the Quadram Institute. A special thanks go to Mark Philo, who was a tremendous help in this process. Bile acids were measured in the scrapings and content of the three different parts of the small intestine, liver, colon faeces and serum. Here for, 50 mg (liver), 25 mg (intestine) or 50 µL (serum) of tissue was homogenised in 1 ml of cold 70% methanol for 30 seconds at

6000 rpm in a Precellys®24 (Bertin Technologies, France). After centrifugation (5 min, 3000g, 4˚C) the supernatant was transferred to a new collection tube and MeOH content was removed by rotary evaporation (70 min, 50˚C). Volume was restored to 1ml by adding 5% MeOH. To clean up the samples they were loaded onto Waters OASIS PRIME HLB 1 30mg SPE cartridges, washed with 5% MeOH and eluted in 500 µl 100% MeOH.
Internal standards were added at the following time points: before homogenisation, before rotary evaporation, before loading onto cartridges, after elusion.
Cleaned-up extracts were analysed using HPLC – mass spectrometry operated in multiple reaction monitoring (MRM) mode.
Each sample (5 µl) was analysed using an Agilent 1260 binary HPLC couples to an AB sciex 4000 QTrap triple quadrupole mass spectrometer. HPLC was achieved using a binary gradient of solvent A (Water + 5mM Ammonium Ac + 0.012% Formic Acid) and solvent B (Methanol + 5 mM Ammonium Ac + 0.012% Formic Acid) at a constant flow rate of 600 µl/min. Separation was made using a Supelco Ascentis Express C18 150 x 4.6, 2.7µm column maintained at 40˚C. Injection was made at 50% B and held for 2 min, ramped to 95%B at 20 min and held until 24 minutes. The column equilibrated to initial conditions for 5 minutes.
The mass spectrometer was operated in electrospray negative mode with capillary voltage of -4500V at 550˚C. Instrument specific gas flow rates were 25ml/min curtain gas, GS1: 40ml/min and GS2: 50ml/min.
See appendix 2 for LC-MS conditions and mass fragmentation monitoring values. Quantification was applied using Analyst 1.6.2 software to integrate detected peak areas relative to the deuterated internal standards.

4.2.5 Serum and liver measurements

Cholesterol was determined in the serum using the corresponding liquicolor kits (Human GmbH, Germany) following the manufactures protocol.

Cholesterol and triglycerides were also analysed in the livers using the same kits. Proceeding this, the lipid fraction was extracted following an adjusted version of the Bligh and Dyer method (Bligh and Dyer 1959). In short, 50 mg of liver tissue was homogenized

in 200 µL chloroform and 400 µL methanol. 200 µL distilled water was added and the sample was homogenized again after which the sample was centrifuged for 10 minutes at 3000g. The lower layer was subsequently transferred to a clean Eppendorf tubes and dried overnight. The dried pellet was dissolved in 250 µL 2% Triton X-100 and this was used to perform the colorimetric assay.

4.2.6 Statistics

Statistics have been performed using Graphpad Prism. The statistical tests used are indicated in each figure legend. Power calculations were based on Fgf15 results observed in the 10 parts study and led to a minimum of 5 mice per group being used in each experiment (indicated per figure). Analysis of variance (ANOVA) provides a statistical test of whether the means of groups are equal. When groups were compared over time, a 2-way ANOVA with Bonferroni post-hoc test was used. When three or more groups were compared at a single time point, a 1-way ANOVA was used with Bonferroni post-hoc. Lastly, when two groups were compared at one time point, an unpaired t-test was used to test for significance. $p<0.05$ was considered overall as statistically significant and significance was indicated as ***= $p<0.001$ **= $p<0.01$ *= $p<0.05$.

4.3 Results

4.3.1 Fructo-oligo saccharides (FOS) decrease ileal *Fgf15* expression

In order to investigate the effects of soluble fibres on *Fgf15* expression in the ileum, fructo-oligo saccharides (FOS) were added to the drinking water of HS fed mice for the duration of one week (figure 4.1A). No differences were observed in either body weight (figure 4.1B) or food intake (figure 4.1C). However, a downregulation of *Fgf15* expression was observed in the ileum of these animals (figure 4.1D). This corresponded with a trend toward an increase of hepatic *Cyp7a1* expression (figure 4.1E) and a significant decrease in circulating cholesterol (figure 4.1F).

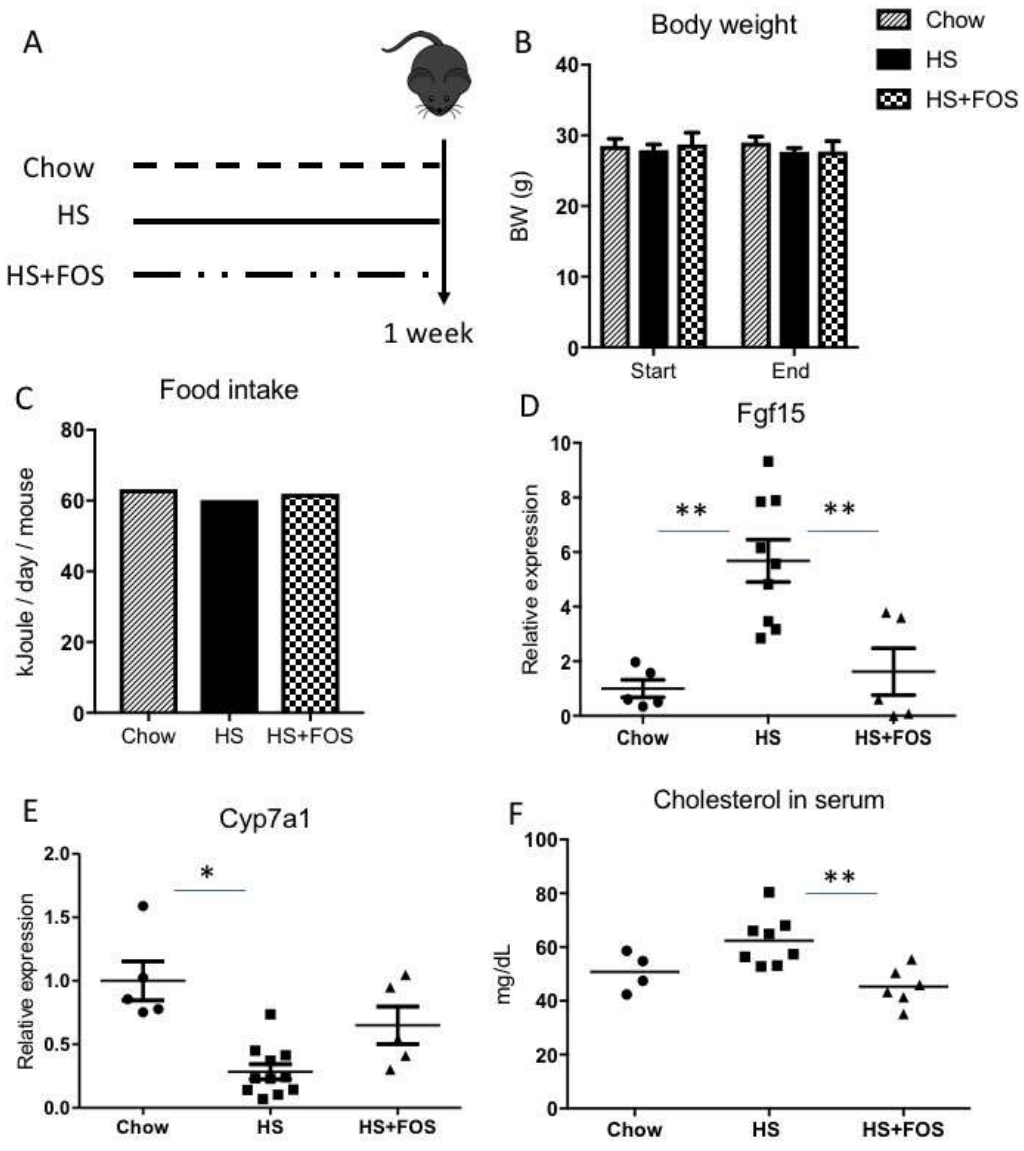

Figure 4.1 FOS in drinking water decreases the expression of Fgf15 in the ileum and consequently lowers blood cholesterol levels. FOS was added into to the drinking water of mice fed the HS diet at a concentration of 30 g/L (A), which did not lead to any changes in either body weight (B) or food intake (C). However, a decrease in Fgf15 was observed in the ileum of FOS treated mice (D), corresponding to a slight trend towards increased Cyp7A1 expression (E) and a significant decrease in blood cholesterol levels (F). *Significance was tested using 1-way ANOVA with Bonferroni post-hoc test between chow and HS diet (*= p<0.05) n≤4 for all groups*

4.3.2 FOS decreases *Fgf15* expression independent of the microbiota

To investigate the role of the microbiota in the downregulation of *Fgf15* expression by FOS, a group of mice was added to the experiment that received the HS diet with FOS in the drinking water and were simultaneously treated with broad spectrum antibiotics for 1 week. Although slightly less pronounced, we observed a similar downregulation of *Fgf15* expression in the antibiotic-treated group compared to conventional mice treated with FOS only (figure 4.2A). Differences in *Fgf15* expression between the non-treated HS group and both FOS treated (with and without antibiotics) groups were statistically significant, differences between the FOS groups were non-significant. *Cyp7a1* expression in the liver was increased in the antibiotic-treated mice compared to all other groups (figure 4.2B), suggesting an additional effect of antibiotics on *Cyp7a1* expression. Taken together, FOS seems to decrease the expression of ileal *Fgf15* independent of the microbiota.

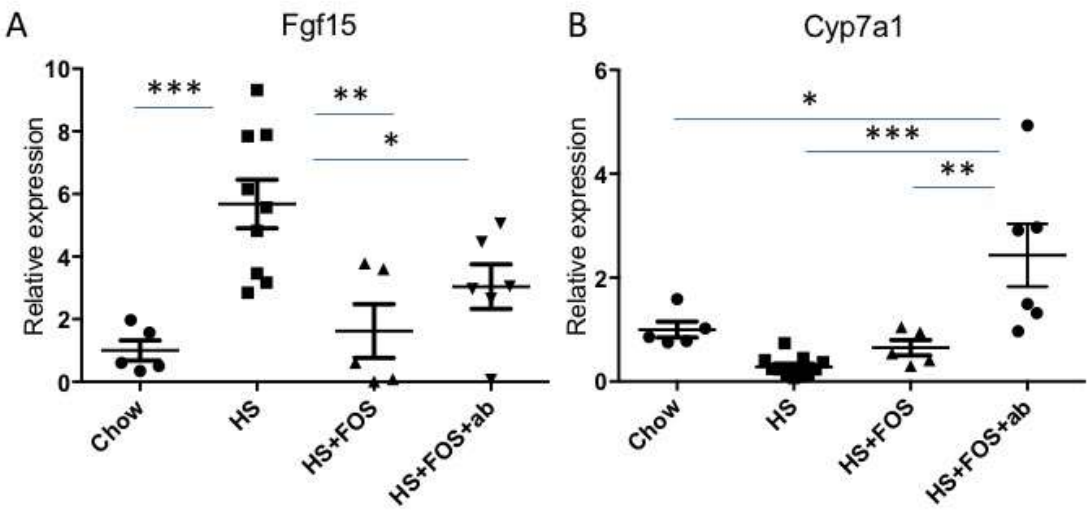

Figure 4.2 The downregulation of Fgf15 by FOS is independent of the microbiota. An additional group of mice was treated with both FOS and broad-spectrum antibiotics on top of the HS diet and showed a similar downregulation of Fgf15 expression (A). In addition, hepatic Cyp7a1 expression was significantly increased in these mice (B). *Significance was tested using a 1-way ANOVA with Bonferroni post-hoc test between all groups (** = p<0.001, **= p<0.01, *= p<0.05). n≤5 for all groups*

4.3.3 Pectin, inulin and fibersol significantly decrease the expression of ileal *Fgf15*

To further investigate the effects that different soluble fibres have on the expression of *Fgf15*, diets were designed containing 75g of soluble fibres per kg of food (figure 4.3A). The first added fibre was inulin, which can be extracted from chicory root and consists of polymers of fructose linked by β-2,1 bonds (Wilson and Whelan 2017). The previously used FOS is similar structurally but contains a shorter chain length than inulin. The second soluble fibre added to the HS diet was pectin, a heteropolysaccharide that can be found ubiquitously in plant cell walls (Naqash et al. 2017). Lastly, fibersol was added to the diets, a non-viscous highly digestion resistant maltodextrin based on corn (Ye et al. 2015). After two weeks of feeding the diets, *Fgf15* expression was quantified in the ileum using q-PCR and was found to be downregulated by all tested fibres (figure 4.3B). A consequent trend towards increased hepatic *Cyp7a1* expression was observed for both pectin and inulin but not fibersol (figure 4.3C). This correlated with a decrease of circulating cholesterol levels, which was only found to be significant for mice fed the pectin diet but a trend towards decreased cholesterol levels was observed for all fibres (figure 4.3D).

A

	HS	Pectin	Inulin	Fibersol
Starch (g/Kg)	506.2	478	478	476.2
Sucrose (g/Kg)	68.8	68.8	68.8	68.8
Cellulose (g/Kg)	50	50	50	50
Soluble fibre (g/Kg)	0	75 Pectin	75 Inulin	75 Fibersol

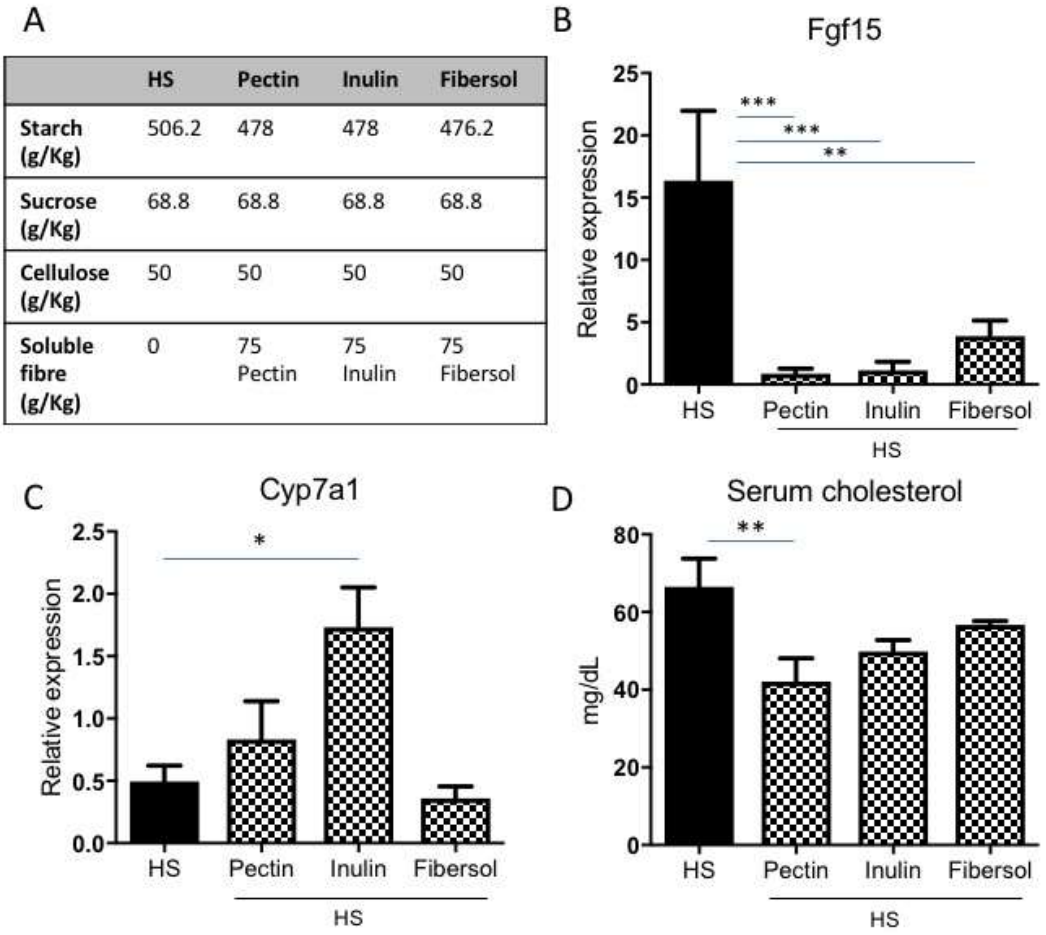

Figure 4.3 All tested soluble fibres decrease ileal Fgf15 expression. Diets were designed to contain 75g of soluble fibres per kg of food, with the different soluble fibres being pectin, inulin and fibersol (A). All diets with added soluble fibres significantly decreased the expression of ileal Fgf15 (B), while hepatic Cyp7a1 was only significantly upregulated by inulin (C). Circulating cholesterol showed a lowering trend on all soluble fibres but was only found significant for pectin (D). *Significance was tested using a 1-way ANOVA with Bonferroni post-hoc test between the HS diet and the other groups (** = p<0.001, **= p<0.01, *= p<0.05) n≤10 for all groups*

4.3.4 Ileal bile acids are unchanged when soluble fibres are added to the diets

To elucidate the molecular mechanisms underpinning the decrease of *Fgf15* expression in mice treated with soluble fibres, we measured bile acids present in ileal tissue. No differences in total bile acids (figure 4.4A) were observed. Upon closer inspection, individual bile acids were also not found to be altered by adding soluble fibres to the HS

diet, although a trend towards increased bile acid content was observed in the fibersol group (figure 4.4B). However, such increases does not easily explain the differences observed in *Fgf15* expression, as an increase in bile acids would lead to an increased expression of *Fgf15* expression (Kuipers, Bloks, and Groen 2014). Additionally, the bile acids are only found to be different in fibersol treated mice, while mice under treatment of all soluble fibres (pectin, inulin and fibersol) showed a decrease in *Fgf15* expression compared to HS fed mice. Therefore, we carefully conclude that the decrease of *Fgf15* expression by soluble fibres is not due to changes in bile acid levels.

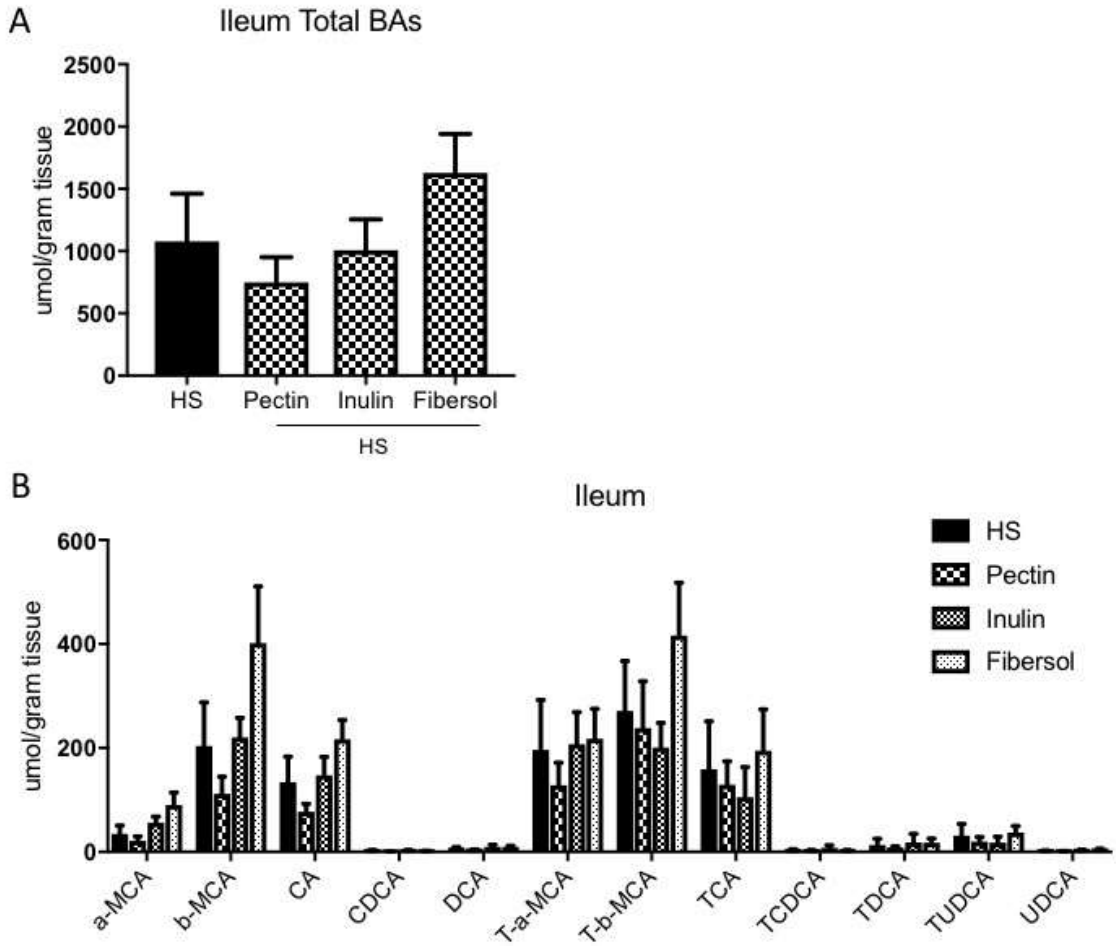

Figure 4.4 Bile acid in the ileum after soluble fibre treatment. No differences were observed in total bile acids present in the ileal tissue after treatment with pectin, inulin or fibersol (A). Individual bile acids also show no significant differences between the diets (B). *Significance was tested using a 1-way ANOVA with Bonferroni post-hoc test between the HS diet and the other groups (** = p<0.001, **= p<0.01, *= p<0.05) n≤10 for all groups*

4.3.5 Bile acid excretion does not increase in response to soluble fibres

Dietary fibres are often hypothesized to prevent the reabsorption of bile acids in the ileum, and thereby increase bile acid excretion (Purnima Gunness and Gidley 2010). To investigate this in the current feeding study, bile acids were quantified in fresh faeces, collected at the time of sacrifice. A trend towards a decrease in bile acids in the soluble fibres diets was observed, mainly for inulin ($p=0.07$) and pectin ($p=0.15$, figure 4.5A). However, no significant differences were observed. A closer look at the individual bile acids revealed a significant decrease in the secondary bile acid DCA after adding pectin or inulin to the diet (figure 4.5B). Since primary bile acids are converted into secondary bile acids by the microbial enzyme BSH (Ridlon et al. 2014), this could indicate an alteration in the microbiota. Although the effect of soluble fibres on *Fgf15* expression seems to be independent of the microbiota, the known effects of fibres on the intestinal microbiota might still be of impact on bile acid profiles. The formation of secondary bile acids mostly takes place in the ceacum and colon (Di Ciaula et al. 2017), and therefor might not influence ileal *Fgf15* expression.

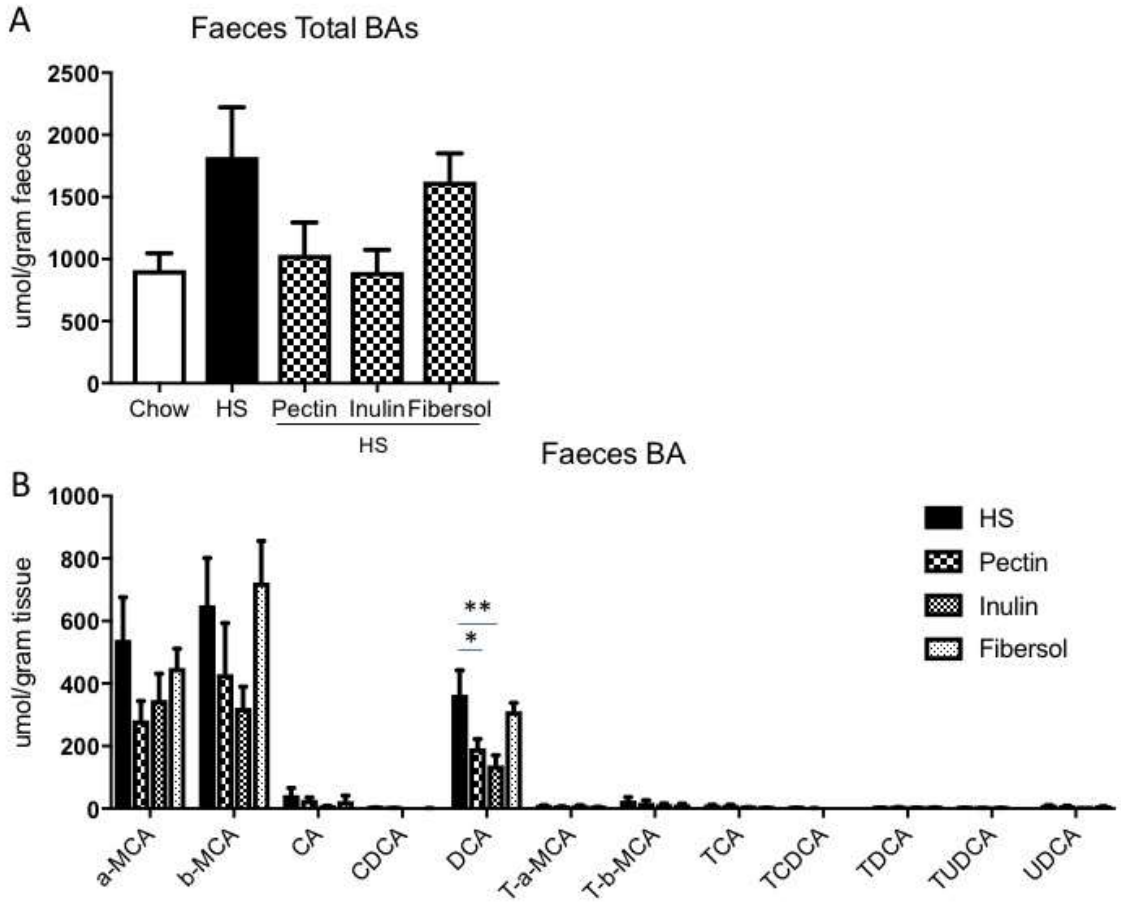

Figure 4.5 Bile acid in the faeces after soluble fibre treatment. No differences were observed in total bile acids present in the faeces tissue after treatment with pectin, inulin or fibersol (A). Individual bile acids show a significant decrease in DCA when pectin or inulin is added to the diet (B). *Significance was tested using a 1-way ANOVA with Bonferroni post-hoc test between the HS diet and the other groups (** = p<0.001, **= p<0.01, *= p<0.05) n≤10 for all groups*

4.3.6 Effects of soluble fibres on glucose metabolism in the ileum

In the previous chapter of this thesis, we outlined how glucose absorption in the ileum can influence *Fgf15* expression. Since soluble fibres have been shown to influence glucose uptake from the intestine (Russell et al. 2016), and have recently even been shown to inhibit intestinal sucrase, an alpha-glucosidase (Neyrinck et al. 2016), soluble fibres could affect *Fgf15* expression by altering glucose absorption. To investigate whether soluble fibres are influencing starch digestion and glucose absorption in the small intestine, we

tested the effects of fibres on glucose metabolism-related genes in the ileum. As a positive control, a group of mice treated with the known α-glucosidase inhibitor acarbose (Rosak and Mertes 2012) was added to the experiment. Mice in this group received the HS diet supplemented with 50 mg of acarbose per kg of BW administered via the drinking water. As acarbose competitively blocks α-glucosidases, we hypothesized a higher gene expression of glucose metabolism-related gene was present in this group.

Glucose uptake from the intestinal lumen into the enterocytes was tested via the expression of glucose transporter 2 (Glut2, *Slc2a2* gene) and sodium-glucose linked transporter 1 (Sglt1, *Slc5a1* gene) (figure 4.6A and B) and the transcriptional induction of α-glucosidases sucrose isomaltase (*Sis*) and maltase glucoamylase (*Mgam*) were tested by q-PCR (figure 4.6C and D). As expected, acarbose treated mice showed increase expression in all glucose metabolism related genes in the ileum. From the fibres, fibersol showed a significant increase in all glucose-related genes (Mgam marginal with p=0.05); while the other fibres show and trend towards a minor increase in *Sis*, *Sglt1* and *Glut2*, but not *Mgam*. Activity measurements of the α-glucosidases will be needed to make stronger conclusions, but for now the results indicate a modest competitive effect of soluble fibres, mostly fibersol, on α-glucosidases activity in the ileum.

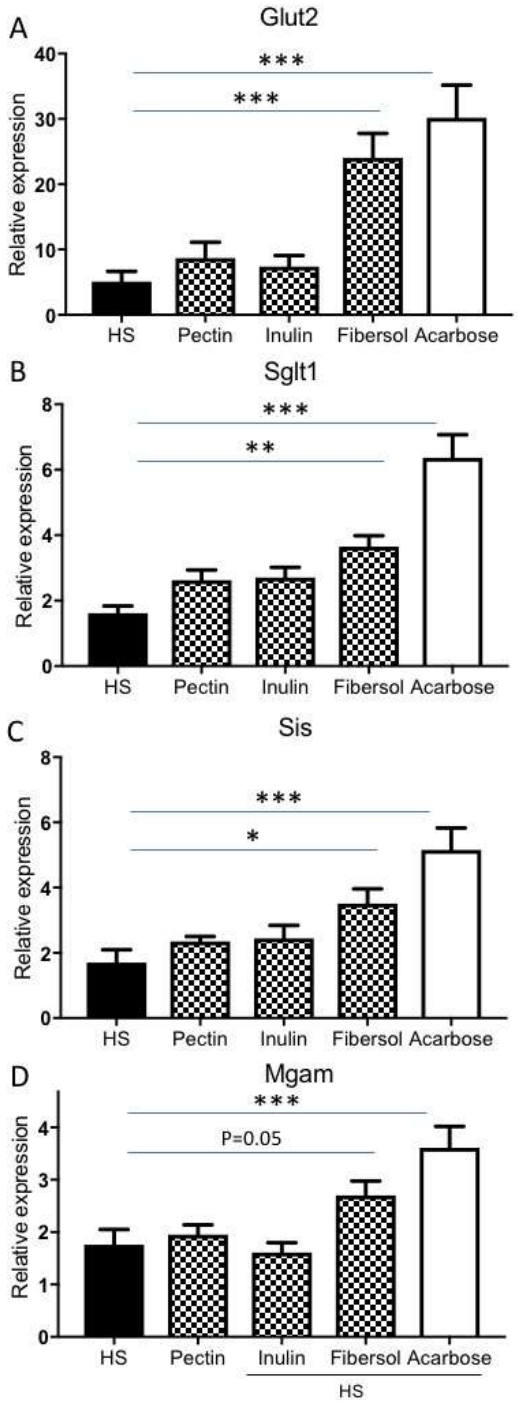

Figure 4.6 Glucose metabolism in the ileum might be affected by soluble fibres. Acarbose was used a positive control for α-glucosidase inhibition. Gene expression the glucose transporters Glut2 (A) and Sglt1 (B) and the α-glucosidases Sis (C) and Mgam (D) was tested via Q-PCR. *Significance was tested using a 1-way ANOVA with Bonferroni post-hoc test between the HS diet and the other groups (** = p<0.001, **= p<0.01, *= p<0.05) n≤10 for all groups*

4.4 Discussion

Here we show that the soluble fibres FOS, inulin, pectin and fibersol decrease the expression of the signalling molecule *Fgf15* in the ileum, and this is most likely independent of the ileal microbiota as shown by the results in the antibiotic-treated mice. The decrease in *Fgf15* expression correlates with an increased expression of *Cyp7a1* in the liver, the enzyme responsible for the conversion of cholesterol to bile acids. Hence, the decreased expression of ileal *Fgf15* might contribute to the known cholesterol-lowering properties of soluble fibres.

FOS are short to medium chain fructose oligosaccharides, and can be produced by the degradation of inulin (Flores-Maltos et al. 2016). FOS and other inulin type of fructans are known to lower total and LDL cholesterol levels (Liu et al. 2017), which we confirm in this study. Additionally, we demonstrate that FOS inhibits the expression of the *Fgf15* in the ileum, and that this effect is more likely independent on the microbiota. Fransen *et al* (Fransen et al. 2017) recently reported a similar decrease of *Fgf15* following FOS intake in germ free animals, supporting that FOS decreases *Fgf15* expression independent of the microbiota. In their experiment, the authors tested both short and medium chain FOS molecules. Although not further investigated by these authors, they reported *Fgf15* being the strongest responding gene to FOS, decreasing its expression by 4-6 fold in germ-free mice. Such effects were observed in conventionally raised mice treated with FOS, however, this could be explained by the small dose used (10mg/day) in combination with bacterial fermentation of FOS. They show by NMR that none of the administered FOS reaches the caecum in conventionally-raised mice (Fransen et al. 2017). Furthermore, Catry *et al* (Catry et al. 2017) found an increase in *Cyp7a1* expression after treating apolipoprotein E knockout mice with inulin like fructans in the drinking water. Although they did not investigate Fgf15 expression in the ileum, the changes in *Cyp7a1* expression are in line with the results presented in this chapter.

Additionally, other soluble fibres were found to decrease the expression of *Fgf15* in a similar manner. Due to the differences in chemical structure of the fibres, these findings were unexpected. Inulin and pectin are both known to reduce blood cholesterol levels (Jiang et al. 2016; Liu et al. 2017); while not much is known about the digestion resistant

maltodextrin fibersol. Here, we show a trend towards a serum cholesterol-lowering effect of fibersol but would suggest longer feeding studies with fibersol to confirm these observations.

Multiple mechanisms have been suggested on how soluble fibres decrease total and LDL cholesterol levels. One of which is the bile acid binding property of soluble fibres. However, this should lead to an increased excretion of bile acids via the faeces, which we did not observe. Others have also questioned the direct binding of bile acids by soluble fibres (Grundy et al. 2016). Furthermore, bile acid excretion from the ileum in ileostomy subjects was not influenced by the addition of inulin or FOS to the diet when compared to sucrose (Ellegård, Andersson, and Bosaeus 2018).

The link between fibres and glucose absorption has been made several times before, with soluble fibres decreasing the uptake of glucose and thereby reducing sugar spikes in the blood (Russell et al. 2016). In addition to this, inulin has been shown to directly inhibit the α-glucosidase sugar isomerase (SIS) in the small intestine (Neyrinck et al. 2016). Mice fed a 5% inulin diet (the diets used in the current study contain 7.5% soluble fibre), showed a significant decrease in SIS activity in the jejunum. This effect was found both in vivo and in vitro suggesting a microbiota independent effect (Neyrinck et al. 2016). SIS is located on the brush border membranes and is responsible for the conversion of di- and oligosaccharides into monosaccharides to facilitate uptake into the small intestinal enterocytes. The most important substrates for SIS are maltose, isomaltose and sucrose (Zagalak and Curtius 1975).

Since *Fgf15* expression was found to be very sensitive to the glucose uptake into the ileum (chapter 3, this thesis), we hypothesize that soluble fibres might inhibit the uptake of glucose from starch in the ileum and thereby decrease the expression of *Fgf15* compared to the HS diet. Unfortunately, we were unable to perform activity test for the α-glucosidases due to shortage of available sample. However, we have tried to obtain some indications by investigating the expression of genes involved in glucose metabolism in the small intestine. If soluble fibres are capable of blocking α-glucosidases, we hypothesized that this would be via competitive inhibition. So, to test this we used an established α-glucosidases inhibitor, acarbose, as our positive control. In the acarbose-treated mice, a clear upregulation of the tested genes (*Sis*, *Mgam*, *Sglt1* and *Glut2*) involved in intestinal

glucose metabolism was observed. In line with literature (Toshinao Goda 2018), the expression of these genes was also increased in mice fed a HS diet. When the additional effect of soluble fibres was tested, we observed an increase in all genes for fibersol and a trend towards a minor upregulation for *Sis*, *Sglt1* and *Glut2* for both pectin and inulin, which could indicate a slight competitive inhibition. Further research will be needed, mostly activity assays, to determine the precise role for dietary fibres on α-glucosidases and thereby starch digestion in the ileum.

In conclusion, soluble fibres decrease the expression of the signalling molecule *Fgf15* in the ileum, which might contribute to the cholesterol-lowering effects of soluble fibres. More research will be needed to further elucidate the molecular mechanisms underlying this inhibition, which should focus on the effects of fibres on glucose absorption in the ileum.

Chapter 5: The role of dietary fibres in the small intestine: impact on the gut-liver axis

Thesis Britt Blokker

Name study	Where	Performed by candidate?	Samples available
10 parts study	WUR	No	Microarray data on intestinal tissue
Soluble fibre study	UEA	Yes	All
Long term study	UEA	Yes	All

In this thesis, we have been comparing a standard chow diet to a semi-purified high starch low fibre diet (HS) and identified major differences in the regulation of the signalling molecule *Fgf15* in the ileum. However, during the course of the experiment, many additional differences were observed in health status pertaining to the gut and liver of these mice. In this chapter, the differences in gut and liver health between the diets are discussed further. Additionally, we try to identify which of these differences are due to the lack of soluble fibres in the HS diet.

5.1 Introduction

In molecular metabolism research there is an increasing number of preclinical studies aiming at identifying the molecular mechanisms underlying the impact of natural and chemical compounds on improving health (Collins and Tabak 2014). Unfortunately, the results of these studies are often not reproducible (Collins and Tabak 2014), at least in part because of the lack of comparable control diets. Currently, the most commonly used control diet is the standard chow diet, a diet composed of partially processed but mostly unrefined plant and animal products. The composition of chow diets can be highly variable which is influenced by factors such as vendor/distributor as well as seasonal and batch variation. Over the years, the chow diet has been proven to be a healthy diet for mice, but its large variability makes it a poor diet for scientific experiments (Pellizzon and Ricci 2018). Furthermore, the chow diet is often used as a control diet in DIO models, after which the effects of the semi-purified high fat diet (HF) are contributed to the fat content of the diet. However, the effects of dietary fat will be confounded by other components, for example the lack of soluble fibres, that differ between diets (Warden and Fisler 2008). This has been demonstrated in the gut microbiota, where it was long believed that the fat content of the diet provoked a switch from primarily *Bacteroidetes* in the gut of chow fed

mice to mostly *Firmicutes* in HF fed mice (Murphy, Velazquez, and Herbert 2015a; Murphy et al. 2010). However, recently it has been shown that this switch in phyla is due to a lack of certain components in the purified diets and not the actual fat content (Dalby et al. 2017).

This problem has been assessed by creating a semi-purified control diet, of which there are several alternatives developed. In 1993, the American Institute of Nutrition developed the AIN-93 diet, an improved version of the original purified control diet developed in 1973 (Reeves, Nielsen, and Fahey 1993). For the AIN-93 diet, dietary fibre content was discussed and set at 50g per kg, with the most important remark being that it should come from a reliable source and that the use of cellulose is suggested. Most purified control diets on the market are based on this diet, and in almost all purified control diets, the dietary fibre content is 50 grams of cellulose per kg. Purified control diets are well defined and consistent in their composition making them suitable control diets. However, it is becoming clear that purified control diets might be detrimental for the mice's health in particular on the long-term, leading to other confounding factors (Pellizzon and Ricci 2018).

The purified diets are composed of sucrose, starch, protein (usually casein) and fat (usually animal-derived lard or plant derived palm oil) with added vitamins and minerals. We hypothesize that one of the most important components missing from the purified diets are soluble fibres. Additionally, we postulate that the amount of sucrose (7-35% versus 5.75% in chow) needs to be reduced to make the control diet more suitable as a healthy control in mouse studies. Previous studies have also indicated that the semi-purified diet lacks a stimulus for the Aryl-Hydrocarbon Receptor (AhR), leading to a decrease in intraepithelial lymphocytes (Ying Li et al. 2011). Furthermore, the AhR has recently been shown to be an important determiner of the metabolic syndrome (Natividad et al. 2018).

Soluble fibres are known to alter the microbiota (Holscher 2017), and a healthy microbiota has been linked to an array of health benefits (Janssen and Kersten 2015). In addition to

alterations in the microbiota, a diet lacking in dietary fibres can alter gut morphology. A low fibre diet has been shown to decrease both the length of the colon and the weight of the ileum in mice. The length of the colon could be restored with short chain fatty acids (SCFAs) feeding, while the caecum weight seemed to be independent of SCFAs production (Chassaing et al. 2015). Although most known effects of fibres are on the colon and colonic microbiota, recently the production of SCFAs from fibres in the small intestine has been reported to be of major importance for the uptake of vitamin A (Goverse et al. 2017).

In this chapter, the aim is to further investigate the effects of the different control diets on the health of mice, with a special interest in the small intestine-liver axis. After feeding the different diets for up to six months, we report alterations in the small intestinal microbiota, gene expression and histology as well as gene expression and histology of the liver.

The second aim of this chapter was to investigate if the negative effects of the purified diet could be counteracted by soluble fibres. A short term study shows promising effects including a less drastic change in the small intestinal microbiota.

5.2 Materials and methods

5.2.1 Murine studies

C56BL6/J mice bred in the DMU of the UEA were used for all murine studies (except WUR study, see below), starting at the age of 10-12 weeks, with a group size of minimum 5 and maximum 10 mice. At the end of the experiments mice were sedated with a mixture of 4% isoflurane (IsoFlo®, Abbott, ND), nitrous oxide (70%) and oxygen (30%) and blood samples were collected via cardiac puncture. The small intestine was collected and arranged in three equal parts, of which the last part was identified as the ileum. Additionally, the liver was collected. All samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis or fixed in 10% formalin (include if you show histology).

All experimental procedures and protocols used in these studies were reviewed and approved by the Animal Welfare and Ethical Review Body and were conducted according to the specifications of the United Kingdom Animals Scientific Procedures Act, 1986.

The WUR study has been performed at the Nutrition, Metabolism and Genomics group of Wageningen University (WUR) in the Netherlands, previous to me commencing my PhD. Here the small intestine was divided into 10 equal parts on which microarrays (Affymetrix GeneChip Mouse Gene 1.1 ST array) were performed. Our group consequently analysed the data using Microsoft Excel.

5.2.2 Diets

Mice were fed either a standard chow diet (RM3-P, Special Diets Services, UK), a HS purified diet (D12450H, Research Diets, USA) for the indicated duration per experiment. WUR mice were fed a standard chow or a HS (D12450H) diet diet for two weeks, however in the WUR diets lard is replaced by palm oil as a fat source. See table 5.1 for a description of the diets used.

Table 5.1 Dietary composition per kilogram of diet. The high starch (HS) diet contains more starch and sucrose and less fibre compared to the chow diet. In the purified diet (HS and high fat (HF) from Research Diets) starch is exchanged for fat.

	Chow diet SDS diets RM3	HS diet D12450H
Energy content	3.35 kcal/g	3.82 kcal/g
Protein	27.28 En%	20 En%
Carbohydrate	61.24 En%	70 En%
Fat	11.48 En%	10 En%
Corn Starch (g)	338.8	452.2
Maltodextrin (g)	0	75
Sucrose (g)	43.7	172.8
Dietary fibre (g)	161.5 Mix of soluble and insoluble	50 Cellulose only
Vitamins See appendix 1	Unknown mix	1 g V10001C
Minerals See appendix 1	Unknown mix	50 g S10026B

5.2.3 Intervention experiments

5.2.3.1 Studies performed at WUR

As explained in Chapter 2, some of the results presented in this work were generated from samples of studies that were performed at WUR. Although these studies were not performed by the PhD candidate, a short summary of the methods used are explained below for the purpose of disclosing all information.

Male C57B6/J mice were fed a standard chow, a low fat (LF, 10 En%) fat) or a high fat (HF, 45En% fat) diet for two weeks (Research Diets, see table 2.1). At the end of the two weeks mice were sacrificed in the fed state and the small intestine was transported to a cold glass plate. Subsequently, the intestine was cut into 10 equal pieces which were

opened and rinsed in phosphate buffered saline (PBS). The cells were scraped out, and RNA was isolated and converted into cDNA. A microarray (Affymetrix GeneChip Mouse Gene 1.1 ST array) was performed at the WUR and data was analysed by our group as outlined in chapter 2.4.

5.2.3.2 Studies performed at UEA / Quadram Institute

The studies outlined below were performed by the PhD Candidate in the DMU of the UEA and samples were analysed at the Quadram Institute. Body weight and food intake was measured once or twice a week for all experiments. Group size is between 5 to 10 mice per group for each experiment. Mice were transferred to a clean cage every week, unless stated otherwise, and were sacrificed in the fed state, unless stated otherwise (see below).

Mice on a chow background diet were fed the chow or HS diet for a duration of 3 days, 1 weeks, 2 weeks, 8 weeks or 6 months before sacrifice.

Antibiotic-treated mice were oral gavaged once daily with a cocktail of vancomycin (50mg/kg BW), Neomycin (100mg/kg BW), Metronidazol (100mg/kg BW) in 0.9% saline. Additionally, mice received ampicillin (1g/L) via drinking water and were transferred to a clean cage daily. This protocol eradicated the microbiota in the ileum, as is shown in figure 3.4D.

The diets used to study the effects of soluble fibres on the small intestine and liver were designed in collaboration with Research Diets Inc (New Brunswick, USA). Briefly, the HS diet (D12450H) was used as a basis from which sucrose levels were reduced to reach similar levels as present in chow (reduced from 172.8 g/kg to 68.8 g/kg, compared to 43.7g/kg in chow). Additionally, 3 more diets were designed containing the soluble fibres pectin, inulin or fibersol (a digestion resistant form of maltodextrin) at a concentration of 7.5%. For more information on the diets see table 5.2.

A concentration of 7.5% was chosen to get close to the amount of soluble fibres present in the chow diet (see appendix 1). The different fibres were selected for their known

prebiotic properties (Wilson and Whelan 2017; Naqash et al. 2017; Ye et al. 2015). While more is known about the properties of both inulin and pectin, Fibersol was selected for its delayed fermentation properties (Ye et al. 2015).

Table 5.2 Dietary composition of the added soluble fibres diets. Rodent diets with 10 En% fat with 50g Cellulose and added with 75g inulin, pectin or soluble corn fibre (Fibersol).

Product #	**D12450J**		**D18012101**		**D18012102**		**D18012103**	
	10 kcal% Fat		*Modified w/*		*Modified w/*		*Modified w/*	
	50 g Cellulose		*50 Cell/75 Inulin*		*50 Cell/75 Pectin*		*50 Cell/75 Fibersol-2*	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	19	*20*	18	*20*	18	*20*	18	*20*
Carbohydrate	67	*70*	62	*70*	62	*70*	62	*70*
Fat	4	*10*	4	*10*	4	*10*	4	*10*
Total		*100*		*100*		*100*		*100*
kcal/gm	3.85		3.68		3.68		3.69	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*	3	*12*	3	*12*
Corn Starch	506.2	*2024.8*	478	*1912*	478	*1912*	476.2	*1905*
Maltodextrin 10	125	*500*	125	*500*	125	*500*	125	*500*
Sucrose	68.8	*275*	68.8	*275*	68.8	*275*	68.8	*275*
Cellulose, BW200	50	*0*	50	*0*	50	*0*	50	*0*
Inulin, Orafti HP	0	*0*	75	*113*	0	*0*	0	*0*
Pectin, Tic Gums 1400	0	*0*	0	*0*	75	*113*	0	*0*
Fibersol-2 (Resistant Maltodextrin)	0	*0*	0	*0*	0	*0*	75	*120*
Lard	20	*180*	20	*180*	20	*180*	20	*180*
Soybean Oil	25	*225*	25	*225*	25	*225*	25	*225*
Mineral Mix S10026	10	*0*	10	*0*	10	*0*	10	*0*
Dicalcium Phosphate	13	*0*	13	*0*	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*
Yellow Dye #5, FD&C	0.04	*0*	0.025	*0*	0	*0*	0	*0*
Red Dye #40, FD&C	0	*0*	0.025	*0*	0	*0*	0.025	*0*
Blue Dye #1, FD&C	0.01	*0*	0	*0*	0.05	*0*	0.025	*0*
Total	**1055.05**	***4057***	**1101.85**	***4057***	**1101.85**	***4057***	**1100.05**	***4057***
gm								
Total Fiber	50.0		125.0		125.0		125.0	
Insoluble Fiber	50.0		50.0		50.0		50.0	
Soluble Fiber	0.0		75.0		75.0		75.0	
gm%								
Total Fiber	4.7		11.3		11.3		11.4	
Insoluble Fiber	4.7		4.5		4.5		4.5	
Soluble Fiber	0.0		6.8		6.8		6.8	
Fiber contents listed above are estimates based on these fiber sources containing 100% fiber and either 100% as soluble or insolu								
Assayed levels: Cellulose = 93%, Inulin = 96%, Pectin = 93%. Fibersol-2 = 90% (manufacturer data)								
Inulin contains 1.5 kcal/gm as per Roberfroid, 1999 (J. Nutr. 129: 1436S–1437S, 1999)								
Pectin doesn't have a specified kcal/g value, so have chosen 1.5 kcal/g to match inulin. MAP								
Fibersol-2 has 1.6 kcal/g according to information from ADM/Matsutani								

5.2.4 Intestinal permeability

In both the 6 months feeding study and the soluble fibre study, intestinal permeability was determined via fluorescein isothiocyanate (FITC) dextran. FITC dextran with a molecular weight of approximately 4 kDa (Sigma-Aldrich, FD4) was administered to the mice via oral gavage at a concentration of 44mg per 100g body weight 2 hours before sacrifice. Mice were sacrificed via cardiac puncture as discussed previously (section 2.1) and whole blood was collected in an Eppendorf tube and kept on ice in the dark. 30 minutes later, the blood was centrifuged for 30 minutes at 3,000g in a refrigerated microcentrifuge and serum was separated out into a clean Eppendorf tube. An aliquot of the serum was subsequently diluted in an equal volume of PBS and 100 µl of diluted serum was added to a 96-well microplate in duplicate. Additionally, a standard serial dilution of FITC-dextran (0, 125, 250, 500, 1,000, 2,000, 4,000, 6,000, 8,000 ng/ml) was added to the 96-wells plate. FITC concentration was determined in a spectrophoto-fluometry with an excitation of 485 nm and an emission wavelength of 528 nm. Serum from mice not administered with FITC-dextran was used to determine the background.

5.2.5 Gene expression

RNA of liver and intestinal scraping samples was isolated. Samples were homogenised in 1 ml Trizol (Qiazol, Qiagen, UK) for 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France). After homogenization samples were transferred to a new Eppendorf tube and 200 µl Chloroform was added. Samples were centrifuged for 15 minutes at 12.000 RCF 4˚C and the clear layer was transferred to a new collection tube. Iso-propanol was added and after mixing, resting for 5-10 minutes and centrifugation for 10 minutes at 12.000 RCF 4˚C an RNA pellet was left. The pellet was washed in 85% ethanol and dissolved in 100 µl RNase Free water. RNA concentration was measured with a Nanodrop (Thermo Scientific, Wilmington, USA). cDNA was synthesized from 2 µg of RNA which was first treated with DNase (DNase kit, Invitrogen, 18068-015) and subsequently reverse transcription was performed. A quantitative polymerase chain reaction (Q-PCR) was performed using SYBR green master mix (Applied Biosystems, Thermo Fisher Scientific, UK) according to the producer's instructions. Reactions were performed using 384 wells (Applied Biosystems, Thermo Fisher Scientific, UK) using

5µL of SYBR green and primer mix (for a list of the primers used, see table 5.3) and 1.5µL of cDNA. The reaction was initialized at 50°C for 5 minutes and 95°C for 2 minutes, after which 40 cycles of denaturation (95°C 15 seconds) and annealing/extension (59°C 1 min) were performed. Afterwards, a melting curve was created and checked for a single product per gene. ΔCT values were calculated by subtracting the CT value of the housekeeping gene TBP from the gene CT value. ΔΔCT was calculated relative to control samples (Livak and Schmittgen 2001).

Table 5.3 Primers used for q-PCR measurements in small intestine and liver.

Gene	Forward	Reverse
TBP (hk)	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT
GAPDH (hk)	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
FXR	GGCCTCTGGGTACCACTACA	AAGAAACATGGCCTCCACTG
OSTA	TTGTGATCAACCGCATTTGT	CTCCTCAAGCCTCCAGTGTC
ASBT	TGGAATGCAGAACACTCAGC	GCAAAGACGAGCTGGAAAAC
FABP6	CACCATTGGCAAAGAATGTG	AACTTGTCACCCACGACCTC
SHP	TCTGCAGGTCGTCCGACTATT	AGGCAGTGGCTGTGAGATGC
FGF15	CAGTCTTCCTCCGAGTAGCG	TGAAGACGATTGCCATCAAG
CYP7A1	GAGCGCTGTCTGGGTCACGG	GCCAGCCTTTCCCGGGCTTT
CYP27A1	TGTGGACAACCTCCTTTGGG	CCATAGGTGAGGCCCTTGTG
CYP8B1	TTGCAAATGCTGCCTCAACC	TAACAGTCGCACACATGGCT

Selected samples went for microarray analysis. After RNA isolation as described before, samples were send to WUR for microarray analysis. At WUR RNA quality was assessed with a Bioanalyzer (Aligent 2100 Bioanalyzer, Santa Clara, USA), and microarrays were performed using the Mouse gene Chip 1.1 array from Affymetrix (Thermo Fisher Scientific, Santa Clara, USA). After normalization process, ~22 k genes were included in the data set. The limma R library was used to analyse the significant differential expression between the diets. Log2FC of 1.5 and a p-value of 0.05 were used as cut-off points and the rate discovery false for the p-value was calculated. Ingenuity Pathways

Analysis (IPA) web-based software application was used to analyse, integrate and interpret the data

5.2.6 Histology

From each UEA mouse, a small part of the liver and a piece of each of the three parts of the small intestine was collected in 10% formalin. 24 hours after collection, these samples were transferred into 50% ethanol and within a week were processed on the Leica ASP 300, program overnight no formalin. The samples were embedded in wax and cut into 5µm sections. Sections were stained with a haematoxylin and eosin (H&E) staining and pictures were taken with an Olympus BX60 microscope.

5.2.7 Caspase 3 activity

For protein extraction, approximately 3 mg of frozen tissue was homogenised on ice in 600 µL AFC lysis buffer (see table 5.4). The homogenate was centrifuged for 10 minutes at 12000 rpm and 4°C. The supernatant was transferred into a new tube and stored at -20°C until used.

Table 5.4 AFC lysis buffer composition for caspase 3 activity.

AFC-Lysis buffer	Volume	Final concentration
Hepes 1M pH 7.4	100 µL	10 mM
Chaps 10%	100 µL	0.1%
EDTA 0.5mM pH 8	40 µL	2 mM
DTT 1M	50 µL	5 mM
dH2O	9.71 ml	

Caspase 3 is a key enzyme for the induction of the apoptotic cascade and is therefore used as a marker of apoptosis. This assay works with a Caspase-3 specific substrate DEVD and a fluorochrome AFC. Active caspase specifically cleaves the substrate DEVD-AFC, thus releasing the fluorogenic AFC which can be quantified by U.V. spectrofluorometry. These tetrapeptide substrates are used to identify and quantify Caspase-3 activity in apoptotic protein lysates.

Per reaction tube, 480 µL mastermix and 20 µL proteins were mixed. Of this mixture 200µL was pipetted into a 96-wells plate in duplicate and incubated at 37°C while gently shaking. Mastermix with AFC-lysis buffer was taken as a blank. AFC-release was measured at 0h, 1h and 2h.

Table 5.5 Mastermix and reaction buffer composition for caspase 3 activity.

Mastermix	Volume/reaction
25x Reaction buffer	20 µL
Ac-DEVD-AFC substrate	2.5 µL
dH2O	457.5 µL
Protein	20 µL

25x Reaction buffer	Final concentration
Pipes 1M pH 7.4	2.5 ml
EDTA 0.5mM pH 8	1 ml
Chaps 10%	2.5 ml
DTT 1M	1.25 ml
dH2O	2.75 ml

5.2.8 Serum and liver measurements

Cholesterol and triglycerides were determined in the serum using the corresponding liquicolor kits (Human GmbH, Germany) following the manufactures protocol.

Cholesterol and triglycerides were also analysed in the livers using the same kits. Proceeding this, the lipid fraction was extracted following an adjusted version of the Bligh and Dyer method (Bligh and Dyer 1959). In short, 50 mg of liver tissue was homogenized in 200 µL chloroform and 400 µL methanol. 200 µL distilled water was added and the sample was homogenized again after which the sample was centrifuged for 10 minutes at 3000g. The lower layer was subsequently transferred to a clean Eppendorf tubes and dried overnight. The dried pellet was dissolved in 250 µL 2% Triton X-100 and this was used to perform the colorimetric assay.

5.2.9 Bile acid measurements

As no bile acid measurements were performed in house at the time of arrival, we set up our own method in collaboration with the metabolomics unit of the Quadram Institute. A special thanks go to Mark Philo, who was a tremendous help in this process. Bile acids were measured in the scrapings and content of the three different parts of the small intestine, liver, colon faeces and serum. Here for, 50 mg (liver), 25 mg (intestine) or 50 µL (serum) of tissue was homogenised in 1 ml of cold 70% methanol for 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France). After centrifugation (5 min, 3000g, 4˚C) the supernatant was transferred to a new collection tube and MeOH content was removed by rotary evaporation (70 min, 50˚C). Volume was restored to 1ml by adding 5% MeOH. To clean up the samples they were loaded onto Waters OASIS PRIME HLB 1 30mg SPE cartridges, washed with 5% MeOH and eluted in 500 µl 100% MeOH.

Internal standards were added at the following time points: before homogenisation, before rotary evaporation, before loading onto cartridges, after elusion.

Cleaned-up extracts were analysed using HPLC – mass spectrometry operated in multiple reaction monitoring (MRM) mode.

Each sample (5 µl) was analysed using an Agilent 1260 binary HPLC couples to an AB sciex 4000 QTrap triple quadrupole mass spectrometer. HPLC was achieved using a binary gradient of solvent A (Water + 5mM Ammonium Ac + 0.012% Formic Acid) and solvent B (Methanol + 5 mM Ammonium Ac + 0.012% Formic Acid) at a constant flow rate of 600 µl/min. Separation was made using a Supelco Ascentis Express C18 150 x 4.6, 2.7µm column maintained at 40˚C. Injection was made at 50% B and held for 2 min, ramped to 95%B at 20 min and held until 24 minutes. The column equilibrated to initial conditions for 5 minutes.

The mass spectrometer was operated in electrospray negative mode with capillary voltage of -4500V at 550˚C. Instrument specific gas flow rates were 25ml/min curtain gas, GS1: 40ml/min and GS2: 50ml/min.

See appendix 2 for LC-MS conditions and mass fragmentation monitoring values. Quantification was applied using Analyst 1.6.2 software to integrate detected peak areas relative to the deuterated internal standards.

5.2.10 Metabolomics

Faecal extracts were prepared for NMR analysis by mixing thoroughly 20 mg of frozen faecal material with 1 mL of saline phosphate buffer (1.9 mM Na2HPO4, 8.1 mM NaH2PO4, 150 mM NaCl, and 1 mM TSP (sodium 3-(trimethylsilyl)-propionate-d4)) in D2O (deuterium oxide), followed by centrifugation (18,000g, 1 min). Supernatants were removed, filtered through 0.2 μm membrane filters, and stored at −20 °C until required. Serum samples were diluted 1:12 with NMR buffer and stored at -20 °C until further analysis. Samples were thawed, and 600 μL of each filtrate was transferred to a 5 mm o.d. NMR tube for analysis.

High resolution 1H NMR spectra were recorded on a 600 MHz Bruker Avance spectrometer fitted with a 5 mm TCI cryoprobe and a 60 slot autosampler (Bruker, Rheinstetten, Germany). Sample temperature was controlled at 300 K. Each spectrum consisted of 128 scans of 32 768 complex data points with a spectral width of 14 ppm (acquisition time 1.95 s). The noesypr1d presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 2 s) and mixing time (D8 = 0.15 s). A 90° pulse length of 8.8 μs was set for all samples. Spectra were transformed with 1 Hz line broadening and zero filling, manually phased, and baseline corrected using the CHENOMX software. Metabolites were identified using information found in the literature or on the web (Human Metabolome Database, http://www.hmdb.ca/) and by use of the 2D-NMR methods, COSY, HSQC, and HMBC (Le Gall et al. 2011).

5.2.11 Microbiota analysis

5.2.11.1 microbial DNA isolation from SI content

The content of each part of the small intestine was gently pushed out of the intestine at the time of sacrifice and rinsed out using 200μ of PBS. 150μL of this was used to isolate microbial DNA from. In collaboration with the Hall lab (Quadram Institute, Norwich Research Park, UK), for DNA isolation we used the Qiagen DNA mini kit following an improved protocol with additional steps to ensure breakage of all bacterial samples kindly provided by Dr. M Lawson. Briefly, the samples were homogenised using glass beads for

4x 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France) and heated to 95°C for 5 minutes in the middle. Additionally, samples were incubated with a lysis buffer containing 20mg/ml lysozyme after which the homogenising was repeated. Consequently, DNA was isolated using the Qiagen DNA mini kit following instruction from the producer. DNA quantity was assessed using both a nanodrop and a Qubit reader.

5.2.11.2 16S sequencing of the SI microbiota

A minimum of 50 ng of DNA was sent to the Earlham Institute (Norwich Research Park, UK) for quality assessment and 16S sequencing using the Illumina platform. Results were further analysed in collaboration with Wiktor Jurkoswki, PhD and Sarah Bastkoswki, PhD from the Earlham Institute.

The microbiome composition was established using QIIME version 1.9.0 (Caporaso et al. 2010). Paired reads were merged during the Quantitative Insights into Microbial Ecology Qiime 1.9.0 illumina workflow employed on a high performance computing cluster. The mapping file includes the forward and reverse linker primer sequence and was used for demultiplexing. Demultiplexing, quality filter and mapping file validation was carried out as described in the Qiime illumina workflow manual. Operational Taxonomic Units (OTUs) were picked within Qiime using the closed-reference OTU picking protocol with the RDP classifier by searching reads against the Greengenes database version gg_13_8 to the 97% level, and reads were aligned with PyNAST. A representative sequence tree was gained after clustering using Uclust version 1.2.22.

We calculated richness as defined through Chao1 (Chao A, (2000)) and diversity as defined by Shannon (Shannon C, (1948)) using the R package Phyloseq (version 1.18.0) (McMurdie P et al., (2013)). The Shapiro-Wilk test was used to assess normality and showed that the alpha diversity values are not normally distributed. We tested if diets had a significant impact on the mouse microbiomes alpha diversity using the Kruskal-Wallis test. A modified one way ANOVA as implemented in lmPerm (version 2.1.0, Wheeler R E, (2010)) was employed, which uses permutation tests instead of normal theory test, and was used to test for effects diet as covariates.

We used the fitZig function of the R package Metagenomeseq (Paulson et al. 2013) to establish significant differences in abundances of OTUs between groupings including covariates. We considered OTUs that were present in at least 25% of all samples.

5.2.11 Statistics

Statistics have been performed using Graphpad Prism. The statistical tests used are indicated in each figure legend. Power calculations were based on expected differences on gene expression (based on previous differences observed in *Fgf15* expression) and led to a minimum of 5 mice per group being used in each experiment (indicated per figure). Analysis of variance (ANOVA) provides a statistical test of whether the means of groups are equal. When groups were compared over time, a 2-way ANOVA with Bonferroni post-hoc test was used. When three or more groups were compared at a single time point, a 1-way ANOVA was used with Bonferroni post-hoc. Lastly, when two groups were compared at one time point, an unpaired t-test was used to test for significance. $p<0.05$ was considered overall as statistically significant and significance was indicated as ***= $p<0.001$ **= $p<0.01$ *= $p<0.05$.

5.3 Results

The macronutrient composition of the two diets is relatively similar, however there are some major variances in sucrose content (3 times higher in HS), starch content (88 gram higher in HS) and fibre content (no fermentable fibre in the HS diet, figure 5.1A). Additionally, previous studies have pointed towards the lack of AhR ligands in the HS diet (Ying Li et al. 2011). Mice receiving these diets for up to six months showed no difference in body weight gain (figure 5.1B) or any other obvious phenotypic differences. However, as will be outlined in this chapter, significant health-relevant differences in the gut and in particular in the liver of these animals were observed.

A

	Chow diet WUR	HS diet WUR
Protein	26.93 En%	20 En%
Carbohydrate	61.57 En%	70 En%
Fat	11.50 En%	10 En%
Starch	339.2g	427.2g
Sucrose	57.5g	172.8g
Fibre	154.3g Mixed soluble and insoluble	50g Cellulose

B

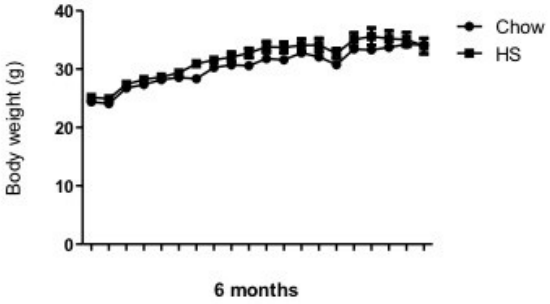

Figure 5.1 Mice fed a chow or semi-purified high starch (HS) diet no not differ in body weight over time. The most important differences between the chow and HS diet are the increase in starch and sucrose and the lack of soluble fibres in the HS diet (A). Body weight gain is not different between the two diet groups over a total of 6 months period of feeding (B). *n=5 for all groups*

5.3.1 Gene expression in the small intestine

Significant differences in small intestinal gene expression were detected between mice fed the chow or purified diet, with 499 genes having a fold change >1.5 and adjusted p-value < 0.05, as found by microarray gene expression analysis. The differentially-regulated genes are both up and downregulated with a higher number of differentially regulated genes at the duodenal part of the small intestine compared to the jejunum and ileum (figure 5.2A). Datamining using the Ingenuity Pathway Analysis software resulted in the prediction of a number of upstream regulators / transcription factors to be down and

upregulated in their activities across the different regions of the small intestine (figure 5.2B and C). Most of these transcription factors we analysed did not show changes in gene expression but likely have a reduced transcriptional activity on one diet or increased on the other diet (chow) because of differences in the corresponding ligands resulting in less or more expression of their target genes. Among the transcription factors with a reduced transcriptional activity on the HS diet is the metabolically important Pparα receptor in the duodenum, involved in lipid metabolism, a number of transcription factors involved in cell growth (*Foxf2*), oxidative stress (*Nfe2l2*), detoxification (*Nr1i2*, *Nr2i3*) and immune response (*Stat1*). Transcription factors with increased activity and target gene expression on the HS diet include immune-related (*Irf5*, *Irf7*, *NfatC2*), cell-cell adhesion in the duodenum (*Ctnnb1*), cell cycle (*Srf, Foxm1, Tp53*), histone acetylation (*Ncoa2*) and gluconeogenesis in the ileum (*Foxo1*). The number and importance of the transcription factors differentially regulated (both increased and decreased) between the two diets emphasises the major effects these diets have on small intestinal health.

Individual gene expression analysis showed that glucose uptake genes are higher expressed under HS conditions, an adaptive and physiological response that is in line with the increased sucrose and starch content of the diet. Additionally, genes involved in vitamin A metabolism are differentially regulated, Pparα target genes are downregulated, inflammation related genes like TNF-α and NFκB are upregulated and barrier function and mucus-related genes are differentially regulated between the diets (Figure 5.2D). Lastly, we observed the downregulation of AhR target gene *Cyp1a1* which is in line with the literature (Ying Li et al. 2011) and a down regulation of an important gene in the kynurenine pathway (Indoleamine 2,3-dioxigenase 1 (*Ido1*)) (figure 5.2E).

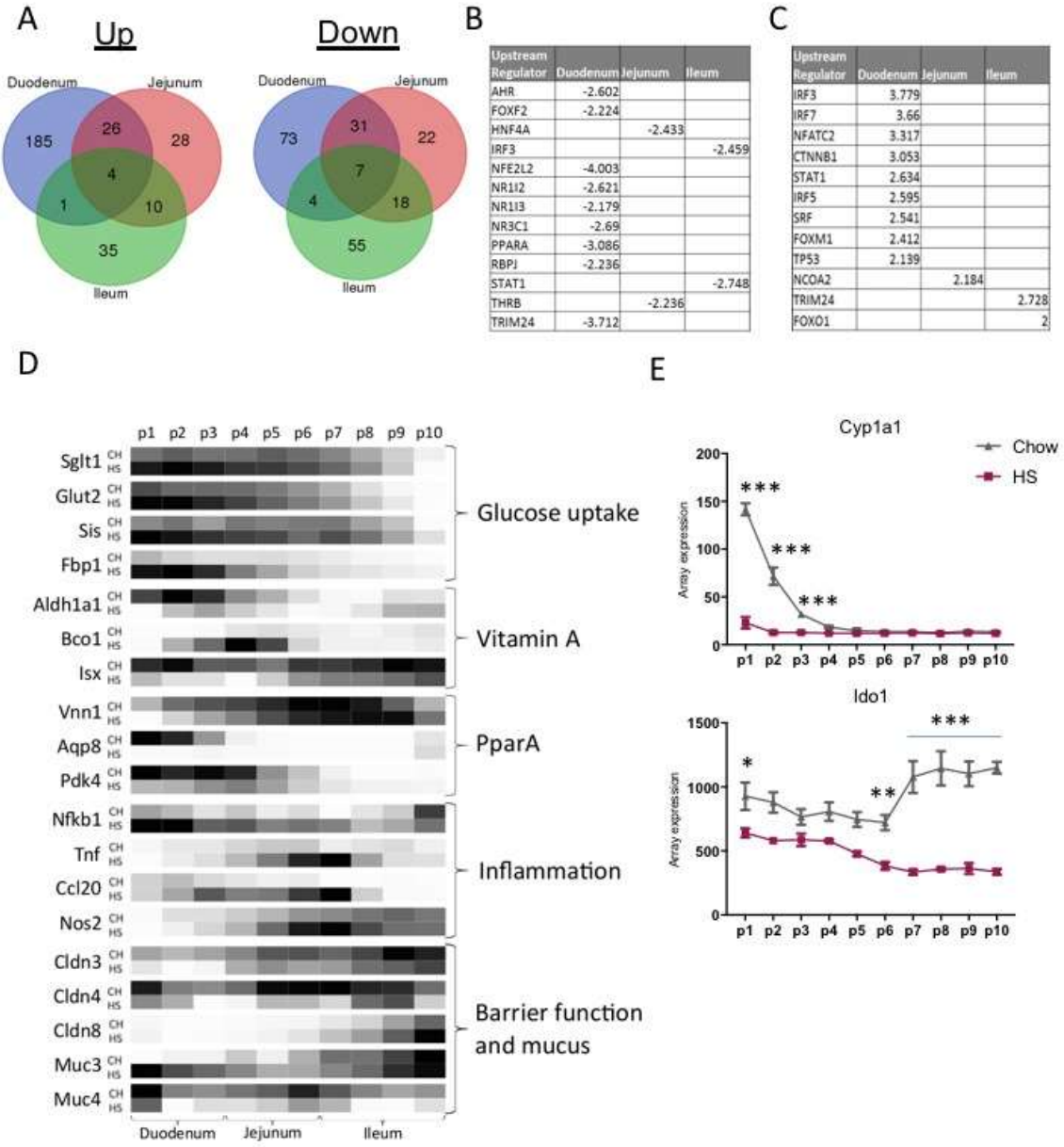

Figure 5.2 Gene expression over the course of the small intestine is dramatically different between chow and HS fed mice. After two weeks of feeding a chow or HS diet, 499 genes were significantly altered over the course of the small intestine, with most genes upregulated in the duodenum of mice fed the HS diet (A). IPA predicted a large number of upstream regulators / transcription factors to be decreased (B) and increased (C) in their activity on the HS diet, and within the pathways individual gene expression was differentially regulated between the diets as shown in expression heatmaps with darker colours indicating higher expression (D). Cyp1a1 expression and Ido1 expression are shown over the course of the small intestine (E). *Significance was tested using or 2-way ANOVA with Bonferroni post-hoc test between chow and the test diets (***= p<0.001, *= p<0.05) n=5 for all groups*

5.3.2 Microbiota in the small intestine

After two weeks of feeding, a switch in microbiota from *Bacteroidetes* to *Firmicutes* was observed in the HS fed mice (figure 5.3A), an alteration that is usually linked to a Western style diet and obesity (Komaroff 2017). Additionally, α-diversity was found to be lower on mice fed the HS diet as shown by a decreased Shannon diversity score (figure 5.3B), indicating less richness in the bacterial community of HS fed animals. Furthermore, on the genus level, a vastly different microbiota profile was found (figure 5.3A), with six significantly different genera between the two diets after multiple testing correction of which five were increased and one was decreased after HS feeding (figure 5.3C). Similar changes were found in a purified HF diet compared to the chow data, indicating that these changes are due to the purification of the diets (data not shown).

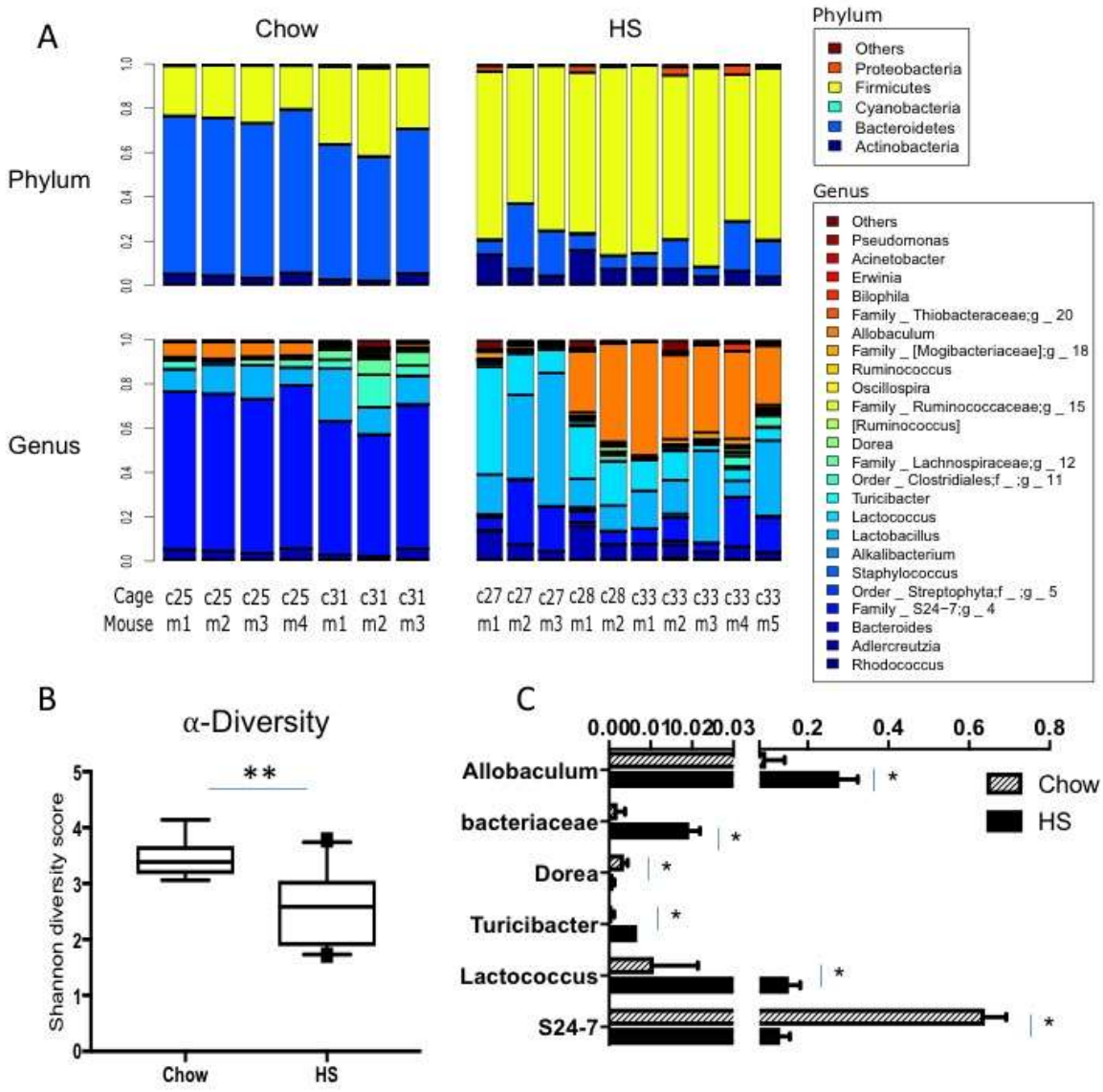

Figure 5.3 Small intestinal microbiota is vastly different after feeding the chow and HS diet. After two weeks of feeding we observed a switch from *Bacteroidetes* to *Firmicutes* in the small intestine at phylum level and different genus profiles were found between the diet, as shown for each individual mouse (A). α-Diversity was calculated via Shannon diversity score and was lower in HS compared to chow fed mice (B). The genera *Allobaculum, Mogibacteriaceae, Dorea, Turicibacterm, Lactococcus* and *S24-7* were significantly different in abundance between the diets (D). *Significance was tested using a student's t-test (**= p<0.01, *= p<0.05) n=5 for all groups*

5.3.3 Metabolomic profiles in the ileum

In addition to 16S-sequencing the microbiota, broad spectrum metabolomics was performed on content from the ileum and caecum of mice fed the chow and HS diet for one week (figure 5.4A). Principle Component Analysist (PCA) revealed a clear difference in specific metabolite concentrations according to diet (figure 5.4B). In particular, large differences in the amino acids present, with an increase of almost all amino acids in the HS fed animals (figure 5.4C) which could indicate a malabsorption of amino acids in the small intestine. Additionally, large variations were found in carbohydrate profiles, with more glucose on the HS diet, but lower amounts of complex carbohydrates (figure 5.4D) which is in line with the absence of soluble fibres in the HS diet. In the caecal content, the increase in amino acids of HS fed mice was also observed (figure 5.4E), stressing the possible malabsorption of amino acids. Furthermore, SCFAs were elevated in the caecum of chow fed mice (figure 5.4F).

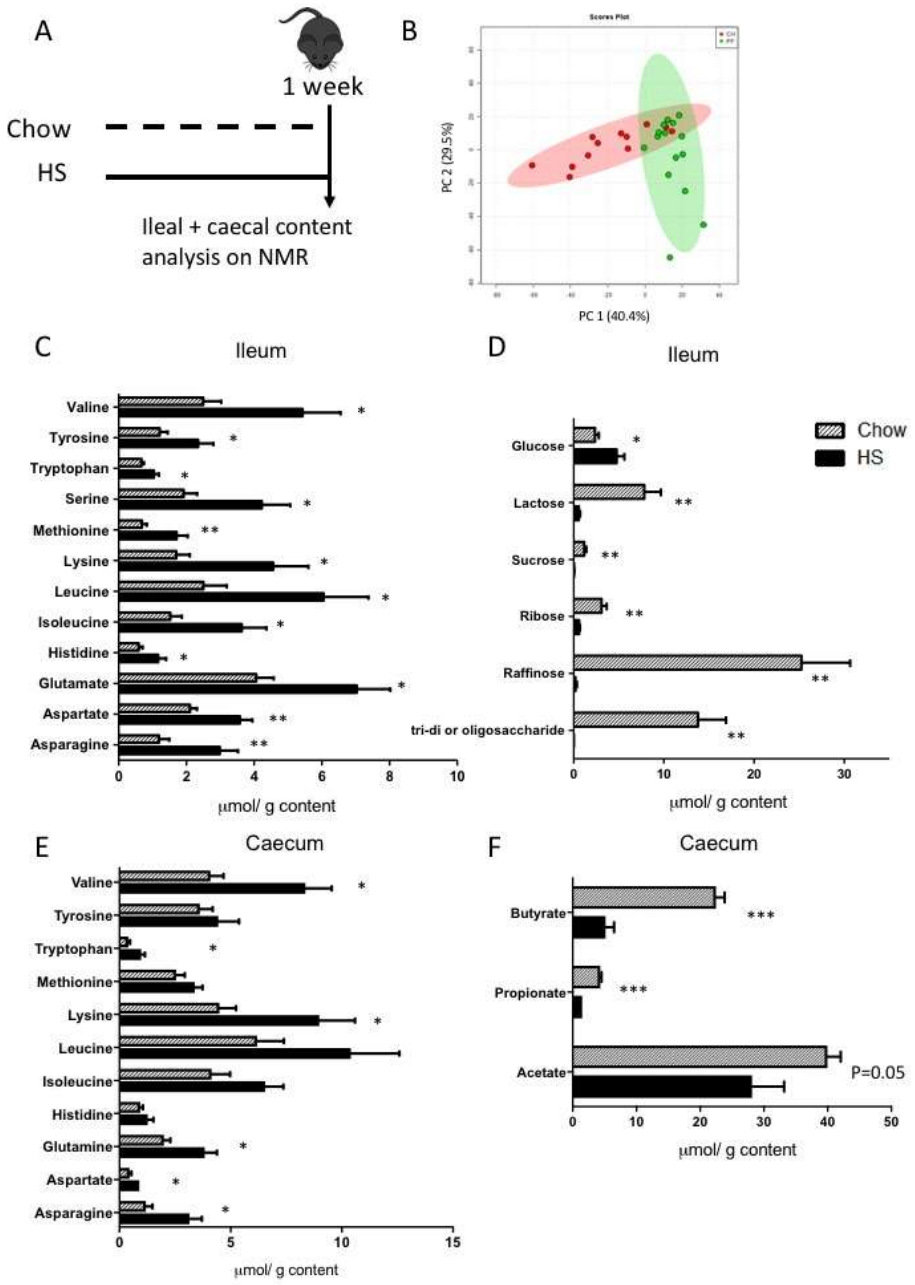

Figure 5.4 Different metabolite profiles isolated from ileum and caecum content between the chow and HS fed mice. After 1 week of feeding, the content from the ileum and caecum was used to extract water-soluble metabolites (A). PCA plot show clear separation of metabolite profiles between the diets (B). In the ileum more amino acids were found in HS fed mice (C), in addition to more glucose and less complex carbohydrates (D). In the caecum, a similar increase in amino acids was observed (E) and a decrease in SCFAs in HS fed mice compared to chow fed mice (F). *Significance was tested using a student's t-test with multiple correction using the Sidak-Bonferroni method (***=p<0.001, **= p<0.01, *= p<0.05) n=5 for all groups*

5.3.4 Ileum metabolite profiles independent of the microbiota

Since large differences in both the microbiota and the metabolite profiles were found between chow and HS fed mice, we investigated the role of the microbiota in influencing ileal metabolite profiles using ^{1}H NMR analysis of the ileal contents of mice treated with broad spectrum antibiotics (figure 5.5A). Here, we observe that also after antibiotic treatment there is a significant alteration of metabolite profiles between chow and HS fed mice (figure 5.5B), indicating direct effects of the diets on the metabolites present in the ileum. In addition, a similar increase in amino acids on the HS fed mice was found (figure 5.5C). Lastly, glucose was still increased in HS fed mice, while other carbohydrates were increased on the chow diet (figure 5.5D). This links back to chapter 3 of this thesis, where glucose reaching the ileum was found to increase the expression of *Fgf15* independently of the microbiota. Different from conventionally-raised mice, no increase in tri-, di- or oligosaccharides was found in antibiotic-treated chow mice, indicating a role of the microbiota in carbohydrate metabolism.

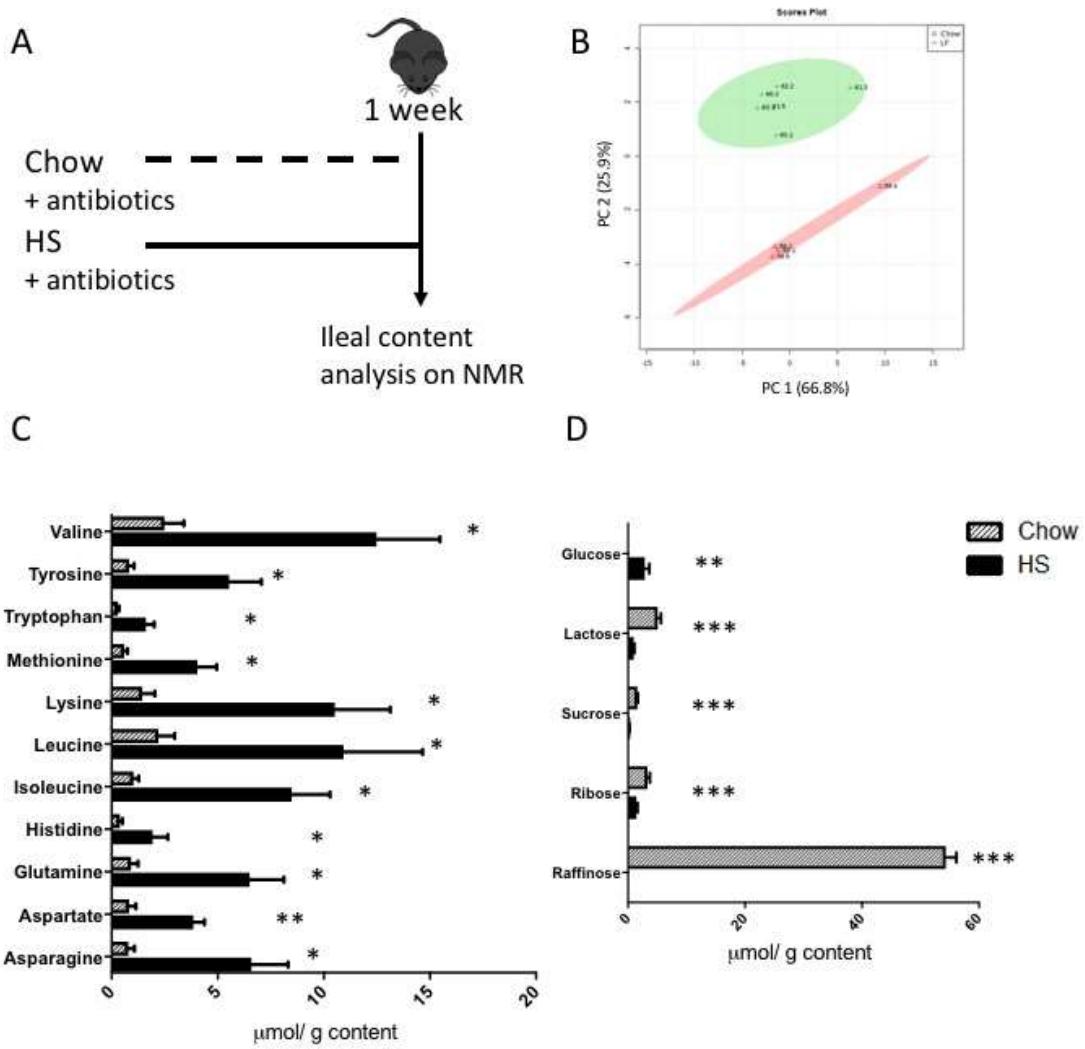

Figure 5.5 Antibiotics treated mice show similar metabolites profiles. Mice were treated with broad spectrum antibiotics while being fed the chow or HS diet, and metabolites were isolated from the content of the ileum (A). PCA plot show an extreme separation between the two diets (B). Again, amino acids were found to be higher in HS fed mice (C), in addition to an increase in glucose and a decrease in more complex carbohydrates (D) *Significance was tested using a student's t-test with multiple correction using the Sidak-Bonferroni method (***=p<0.001, **= p<0.01, *= p<0.05) n=10 for all groups*

5.3.5 Small intestine physiology

To determine the long-term consequences of the observed metabolic changes in the small intestine, a long-term study was performed to compare the effects of the chow and HS diet after six months of feeding. Due to the previously observed alteration in gene expression and microbiota profiles, we hypothesized alterations on small intestinal physiology. Consequently, H&E staining was performed on ileum samples (figure 5.6A) and a shorter villi length was observed in the ileum (figure 5.6B), which is most likely due to the lack of fibres in the LF diet (figure 4A and B) (Sigleo, Jackson, and Vahouny 1984). This difference was only found for the ileum, but not in the duodenum nor in the jejunum, and only after six months of feeding the diets (data not shown). Because of the previously observed differences in barrier function and mucus-related genes, intestinal permeability was tested using FITC-dextran. FITC-dextran was administered via oral gavage two hours before sacrifice and the appearance of fluorescence was measured in the serum. A higher fluorescence indicated an increase in membrane permeability in the intestinal epithelial layer, as FITC dextran is absorbed (see methods 2.4.3). An increase in intestinal permeability was observed in mice fed the HS diet for six months (figure 5.6C), which is linked to a variety of gut and metabolic conditions including obesity and the metabolic syndrome (Bischoff et al. 2014). Additionally, microarray analysis was performed on the ileum of these mice. Genes found to be differentially regulated in the two-week experiment were investigated and mostly also differentially expressed in this long-term experiment. The decrease in *Ido1* and *Cldn3* could be confirmed, as well as the increase in *Nos2* expression (figure 5.6D). Further, glucose metabolism genes were analysed and found to be upregulated in the ileum of HS fed mice (figure 5.6E). This is in line with the previously observed increase in these genes on the HS diet, however, after two weeks the increase seemed to be mostly in the duodenum and jejunum while after six months they could also be observed in the ileum.

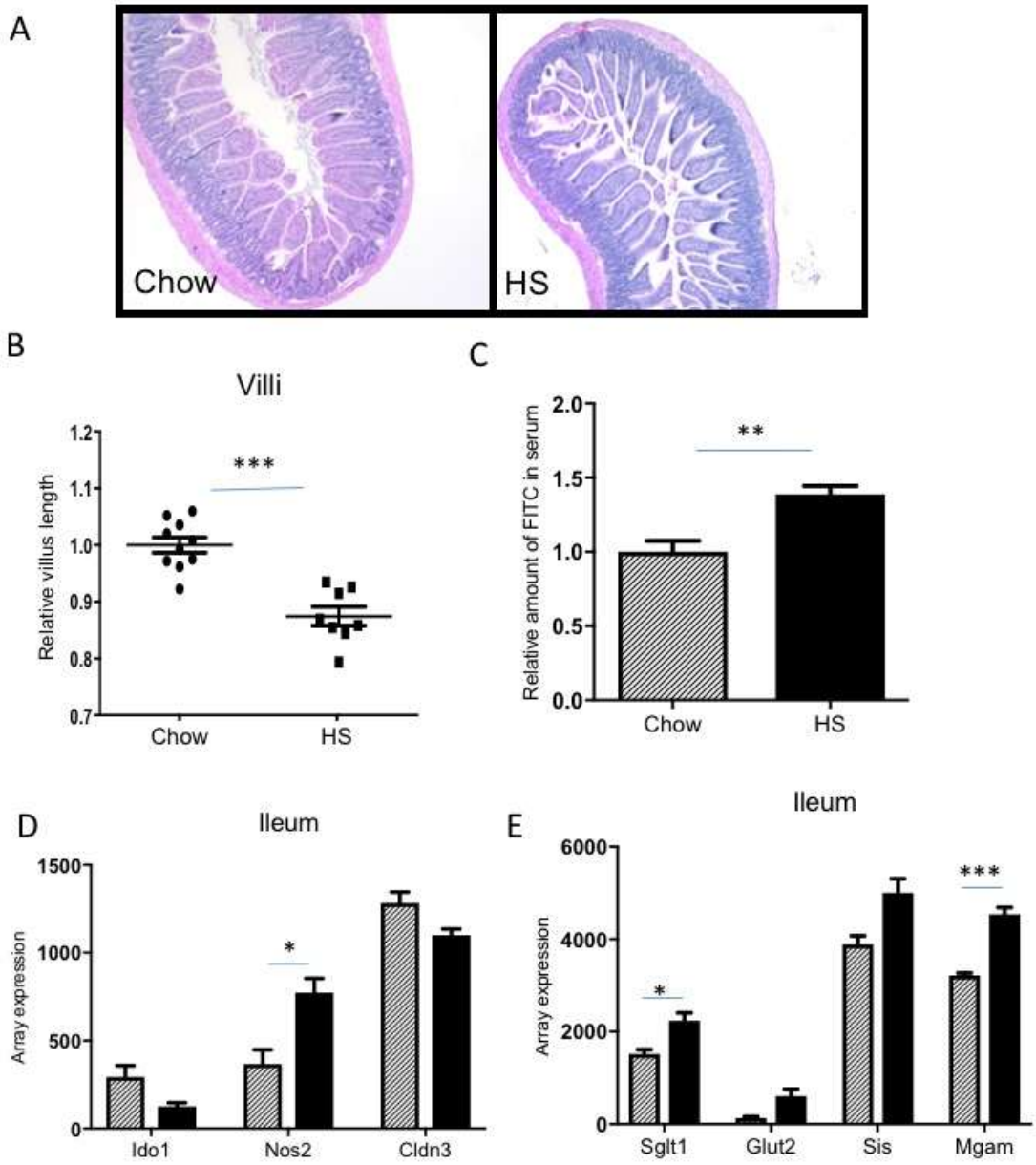

Figure 5.6 Long term feeding of the HS diet decreases villus length and leads to more intestinal permeability. After 6 months of feeding the two control diets, we investigated the small intestinal physiology by H&E staining (A) and found a decrease in villus length in the ileum (B). Additionally, long term feeding if the HS diet increased intestinal permeability as measured by FITC dextran (C). Gene expression, as measured via microarray, showed that similar effects can be found long term on intestinal gene expression as were previously identified short term (D) and additionally glucose metabolism genes are upregulated in the ileum of HS fed mice (E) *Significance was tested using a student's t-test or a t-test with multiple correction using the Sidak-Bonferroni method (***=p<0.001, **= p<0.01, *= p<0.05) n≤9 for all groups*

5.3.6 Consequences for the liver

The intestine and liver together form the gut-liver axis, linked though the enterohepatic circulation of diverse metabolites, which is critical in the development of a variety of health problems including metabolic and liver diseases (Wiest et al. 2017). To investigate the effects of the diets on the liver, H&E staining was performed on livers of mice on these diets over time (figure 5.7A). Short-term and long-term histological differences were observed, showing hepatocyte ballooning which coincided with increased triglyceride levels on the HS diet (figure 5B). In rodents, this coincides cholesterol levels (shown in figure 3.2), while this link is not present in humans and nonhuman primates (Khovidhunkit et al. 2004). In addition, liver damage was quantified by apoptosis determination via caspase 3 activity measurements and showed a trend ($p=0.1$ at 1 week) towards an increase on the HS diet (figure 5.7C). The changes in fat accumulation and tissue damage were also reflected on the gene expression level on the short (figure 5D) and long term (figure 5E). Genes increased in livers of HS fed mice include the fatty acid synthase (*Fasn*), αSMA (gene name *Acta2*) as an early indicator of fibrosis and tumor necrosis factor α (*Tnf*) as an indicator of inflammation. On the long term the HS diet even led to the development of hepatic carcinoma in 2 out of 9 animals, compared to 0 out of 10 chow fed animals (chapter 3, figure 3.10). Interestingly, the diets seemed to have an initial metabolic stress effect on the livers (3 days and 1 week), after which the phenotypes would modestly recover; while on the long term (6 months) the detrimental phenotypes would become more obvious. This is reflected in the liver histology, liver triglyceride quantification and in the gene expression profiles (data not shown).

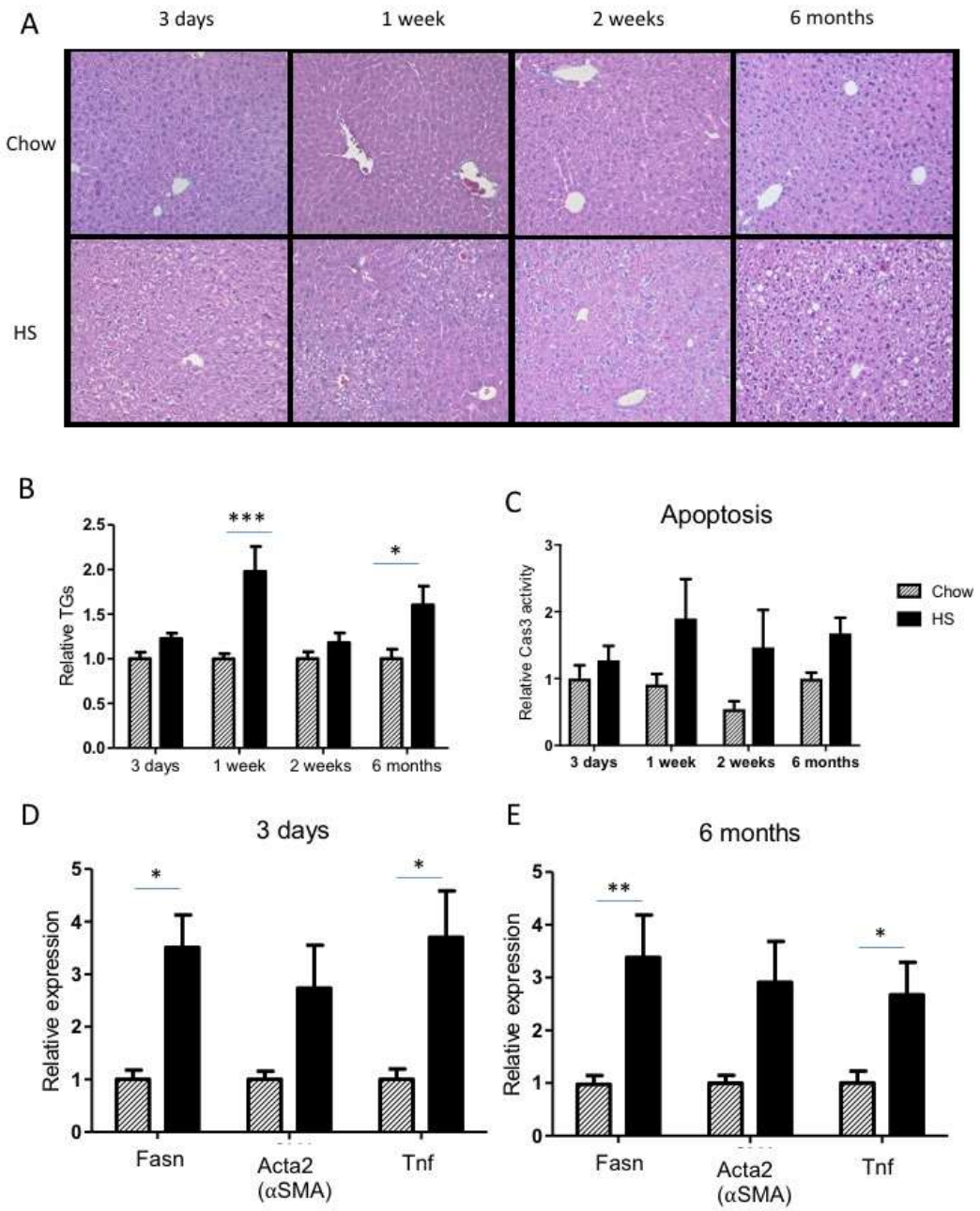

Figure 5.7 The HS diet induces triglyceride accumulation and apoptosis in the liver. H&E pictures of livers of mice fed the chow or HS diets for 3 days to 6 months show more fat accumulation and tissue damage in the HS fed mice (A). The accumulation of triglycerides is significant short term (1 week) and long term (6 months) (C). Apoptosis was measured by caspase 3 activity and shows a trend towards and increase on the HS diet (D). The changes in liver phenotype are also reflected in gene expression profiles after 3 days (E) and 6 months (F). *Significance was tested using a 2-way ANOVA with a Bonferroni post-hoc test or a student's t t-test with multiple correction using the Sidak-Bonferroni method (***=p<0.001, **= p<0.01, *= p<0.05) n≤5 for all groups*

5.3.7 Effects of soluble fibres on the gut-liver axis

As discussed previously, we hypothesized that the lack of soluble fibres in the HS diet may be a major contributor to the phenotypes observed in both the small intestine and the liver. Additionally, we suspected that the sucrose content of the HS diet may also contribute to the pathological liver phenotypes. To test these hypotheses, new diets were designed in collaboration with Research Diets Inc, based on the original HS diet. Firstly, sucrose content was decreased by over 60% to simulate sucrose levels found in the chow diet. Additionally, 75 grams of soluble fibres was added to the HS diet. Firstly, inulin was added, a fibre that can be extracted from chicory root and is a polymer comprising fructose residues linked by β-(2,1) bonds (Wilson and Whelan 2017). The second soluble fibre added to the HS diet was pectin, a hetero-polysaccharide that can be found ubiquitously in plant cell walls (Naqash et al. 2017). Lastly, fibersol was added to the diets, a non-viscous and highly digestion-resistant maltodextrin (Ye et al. 2015). A summary of the compositions of the diets can be found in table 5.6 while a more detailed description is given in the materials and methods table 5.2.

Table 5.6 Dietary composition of diets designed in collaboration with Research Diets.

	Chow diet SDS diets RM3	HS diet D12450H	HS diet Low sucrose	HS diet Pectin	HS diet Inulin	HS diet Fibersol
Protein	27.28 En%	20 En%	20 En%	20 En%	20 En%	20 En%
Carbohydrate	61.24 En%	70 En%	70 En%	70 En%	70 En%	70 En%
Fat	11.48 En%	10 En%	10 En%	10 En%	10 En%	10 En%
Starch (g/Kg)	338.8	452.2	506.2	478	478	476.2
Sucrose (g/Kg)	43.7	172.8	68.8	68.8	68.8	68.8
Dietary fibre (g/Kg)	161.5 Mix of soluble and insoluble	50g cellulose	50g cellulose	50g cellulose + 75g pectin	50g cellulose + 75g inulin	50g cellulose + 75g fibersol

In order to detangle the role of soluble fibres, the HS diet used in this experiment is the low sucrose HS diet. The low sucrose HS diet and the corresponding fibre diets only differ in soluble fibre content, corrected for caloric intake by a slight decrease in starch. No significant differences in body weight or food intake were observed between the diets

(figure 5.8A and B). To investigate the morphology of the gut, we measured the length of the small intestine and colon and the weight of the caecum. In line with previous reports (Chassaing et al. 2015), an increase in caecum weight was observed in the fibre rich diets, however, no differences in small intestinal or colon length were evident (figure 5.8C).

To further investigate which of the previously observed intestinal dysregulations were caused by a lack of dietary fibres, targeted gene expression across the small intestine was investigated using q-PCR. *Cyp1a1* was tested in the duodenum as a target gene of the AhR, which did not respond to the addition of soluble fibres to the diet. Vitamin A metabolism was tested via Beta-Carotene Oxygenese 1 (*Bco1*) expression in the jejunum and showed an improvement in response to soluble fibres, with inulin and pectin normalizing *Bco1* expression completely and fibersol being less potent. Lastly, ileal *Ido1* expression was tested in the ileum and did not show any differential response to the fibres. Intestinal permeability was also tested using oral gavage of FITC dextran as previously described, but no differences between the groups were observed (data not shown).

To investigate the role of soluble fibres on the gut-liver axis, liver histology was performed. To determine the effects of the newly-designed diets on liver histology, a group of mice on the original high sucrose HS diet were included. As predicted, the reduced sucrose content of the new HS diet improved the liver phenotype considerably. The soluble fibres pectin and inulin were found to further improve the phenotype, while fibersol did not appear to have any beneficial effect on liver phenotypes. It has to be considered that this was a short-term study and longer feeding studies are necessary to further decipher the effect of the soluble fibres on liver health.

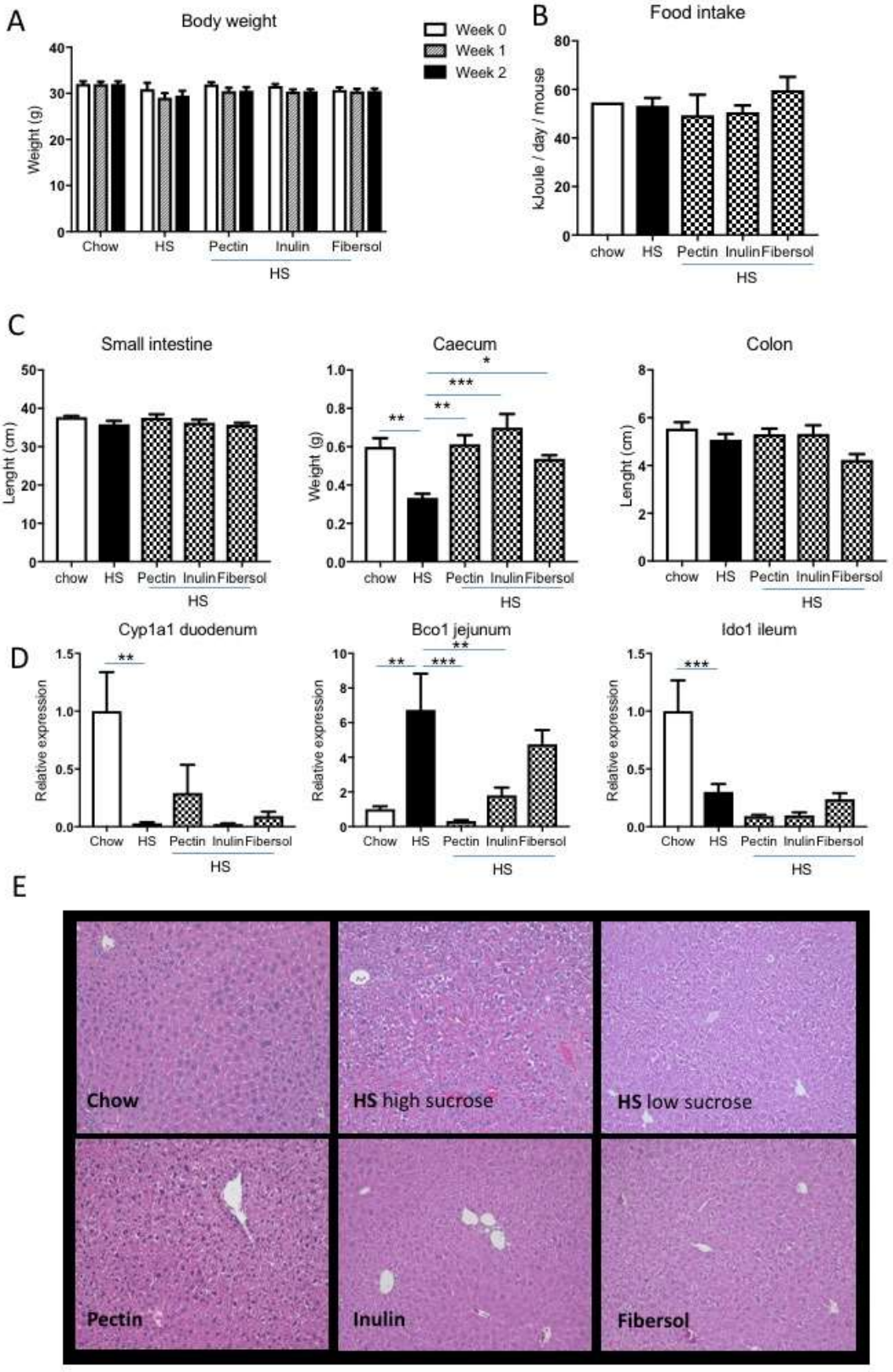
A
Body weight
Week 0
Week 1
Week 2
Weight (g)
Chow
HS
Pectin
Inulin
Fibersol
HS
B
Food intake
kJoule / day / mouse
chow
HS
Pectin
Inulin
Fibersol
HS
C
Small intestine
Lenght (cm)
Caecum
Weight (g)
Colon
Lenght (cm)
D
Cyp1a1 duodenum
Bco1 jejunum
Ido1 ileum
Relative expression
E
Chow
HS high sucrose
HS low sucrose
Pectin
Inulin
Fibersol

Figure 5.8 Adding soluble fibres to the HS diet partly counteracts its detrimental effects on the gut-liver axis. Body weight did not change when the soluble fibres were added to the HS diet (A) and neither did food intake (B). Length of the small intestine and colon and weight of the caecum were measured during sacrifice (C). Gene expression of genes previously found to be differentially expression between the chow and HS diet were tested via Q-PCR in the duodenum, jejunum and ileum (D). Lastly, liver phenotypes were studied by histological H&E staining (E). *Significance was tested using a 2-way ANOVA (A) or 1-way ANOVOA (rest) with a Bonferroni post-hoc test for differences against the HS diet group (***=p<0.001, **= p<0.01, *= p<0.05) n=10 for all groups*

5.3.8 Effects of soluble fibres on the ileal microbiota

Since soluble fibres are known to alter the gut microbiota, 16S sequencing was performed on the content of the ileum to identify changes in the ileal microbiota between the diets. Firstly, α-diversity as calculated by Shannon score was again found to be lower in HS fed mice compared to chow fed mice (also see figure 5.3B), and here it is shown that adding soluble fibres to the HS diet does not increase the microbial diversity in the ileum (Figure 5.9A). Additionally, in figure 5.3A we show that there is a switch from predominantly *Bacteroidetes* in chow fed animals to predominantly *Firmicutes* in HS fed animals. Here, the percental abundance of *Bacteroidetes* is shown to increase after adding pectin and mostly inulin to the HS diet (figure 5.9B). Furthermore, on the phylum level an increase in *Actinobacteria* is shown in the Fibersol group ($p<0.001$ for HS versus HS+Fibersol, figure 5.9C). Lastly, at genus levels microbial profiles are altered by the soluble fibres. For example, an unknown species from the *S24-7* family is decreased in HS fed animals compared to chow fed animals ($p<0.001$) and increased by adding soluble fibres to the HS diet, mostly inulin (HS vs HS+inulin $p<0.001$, Chow vs HS+inulin NS).

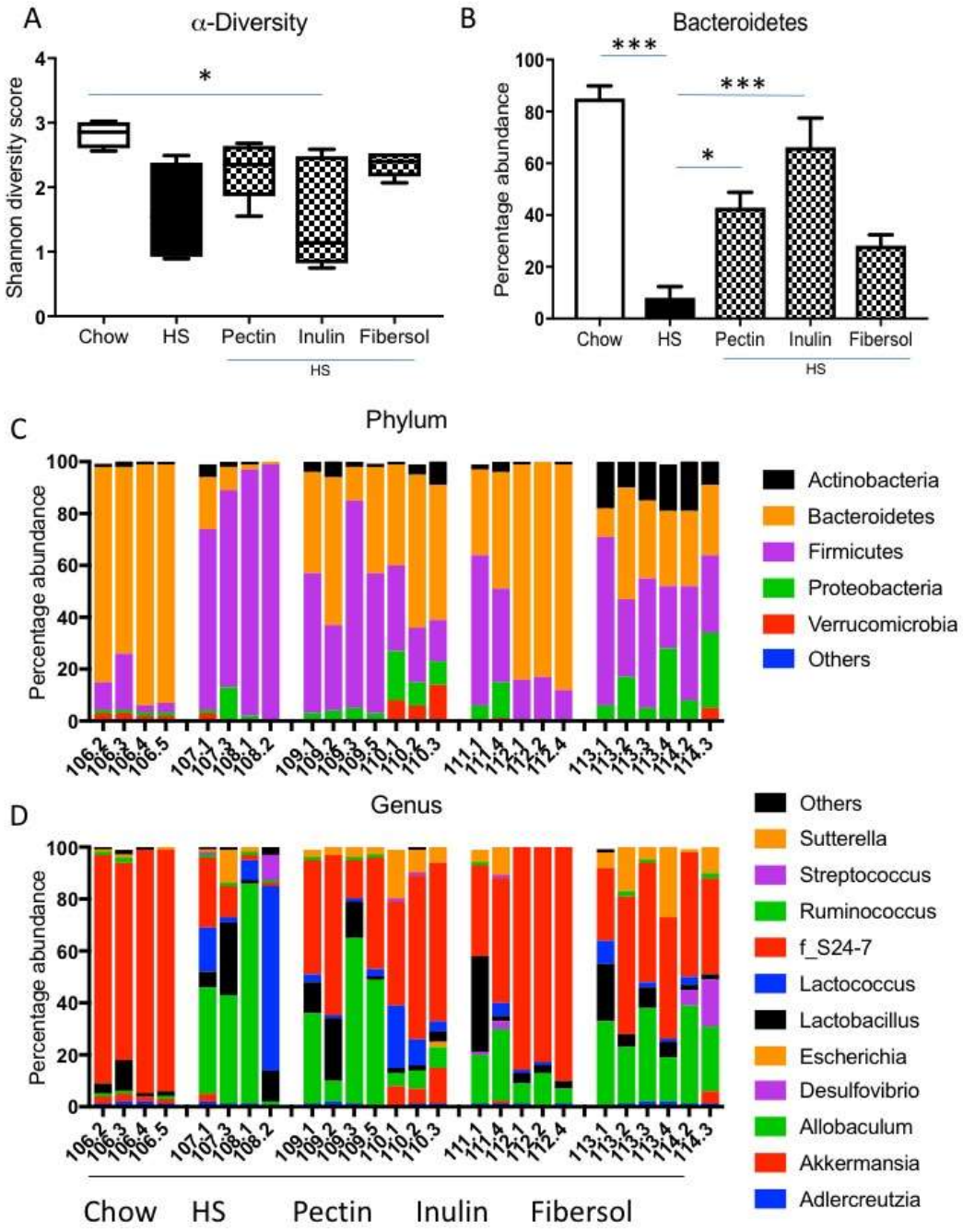

Figure 5.9 Adding soluble fibres to the HS diet partly restored the ileum microbiota. After two weeks of feeding a decrease in microbial diversity as calculated by the Shannon score was observed in all HS fed mice which did not improve after adding the soluble fibres pectin, inulin or fibersol to this diet (A). The Bacteroidetes / Firmicutes ratio was decreased in HS fed animals and slightly increased after adding soluble fibres (B). Phylum (C) and genus (D) profiles show that microbial profiles in the ileum are altered by soluble. *Significance was tested using a one-way ANOVA with Bonferroni post-hoc test (***= p<0.001, *= p<0.05) n≤4 for all groups*

5.4 Discussion

Here it is shown that a diet consisting of purified components compared to an equicaloric chow diet has fundamental effects on the gut-liver axis independent of the development of obesity. The semi-purified HS diet leads to substantial changes in 1. gene expression of metabolic activity in the different regions of the small intestine, 2. the small intestine microbiota with a large increase in *Firmicutes* at the expanse of *Bacteriodetes*, 3. luminal metabolite profiles showing an increase in amino acids and a decrease in SCFAs and 4. leads to pathological liver phenotypes as shown by apoptosis and TG content of the livers. Additionally, we show that these effects are in part due to a lack of soluble fibres highlighting the role of fibre not only in the colon, but also in the small intestine, and that fibres are beneficial for the gut-liver axis. For human diets this would indicate that a diet low on soluble fibres alter the small intestinal microbiota, to a profile that has been shown to be less favourable (Murphy, Velazquez, and Herbert 2015). Additionally, the chow diet can be compared with a more "whole food" diet, and the HS diet with a "processed" diet, emphasising in this chapter that a "whole food" diet is better for the health of both the small intestine and the liver.

No changes in body weight of mice were found between these two diets in this study and by others (Dalby et al. 2017), which indicates that the observed effects here are independent of obesity development. Due to the known differences between the diets, some differences in gene expression in the small intestine were expected as part of a physiological adaptive response. For example, the increase in glucose and fructose uptake genes were increased about three-fold in the HS fed mice when compared to the chow fed mice, which correlated with the three-fold increase in sucrose content of the diet. On a long-term, glucose metabolism genes also changed in the ileum. In addition to these obvious differences, many unexpected differences were encountered. The major differences were mainly observed in the activity of transcription factors and the related regulation of their target genes in the small intestine indicating the vastly different effects the diets and their bioactive components have on the small intestinal immune and metabolic functions.

Additionally, a dysregulation in the vitamin A pathway in the small intestine, indicating a potential decrease in absorption of vitamin A, in HS fed mice was found, which has

previously been described and found to be related to SCFA production in the small intestine (Goverse et al. 2017). We here confirm that adding soluble fibres to the HS diet at least partly restores vitamin A metabolism in the small intestine. Our results indicate a strong effect of soluble fibres on *Bco1*, an enzyme that catalysis the conversion of β-carotene to vitamin A and that is under control of the small intestine specific transcription factor *Isx* (Lobo et al. 2013). Dysregulation of the *Isx-Bco1* pathway has been shown to affect lymphocyte differentiation in the gut as well as increase vitamin A levels in the liver (Widjaja-Adhi et al. 2017). Fibersol showed to be less potent in decreasing *Bco1* expression, which might be related to the previous suggestion that fibersol is fermented in the more distal gut (Fastinger et al. 2008).

Indoleamine 2,3-Dioxygenase 1 (*Ido1*) is the rate limiting enzyme in tryptophan catabolism via the kynurenine pathway which is responsible for 90% of the tryptophan metabolism (Kennedy et al. 2017). The gut microbiota is known to influence tryptophan metabolism, as germ free and antibiotic-treated mice show an increase in circulating tryptophan levels (Wikoff et al. 2009; Desbonnet et al. 2015). Since tryptophan metabolism is essential for the brain, dysregulation of the kynurenine pathway has been linked to a number of psychiatric disorders, including anxiety and depression (Kennedy et al. 2017). In the current study, a decrease of Ido1 expression is observed on the HS diet, which could be caused by changes in microbiota. However, no improvement in *Ido1* expression was found after adding soluble fibres to the diet, indicating that the observed effect is likely not linked to the soluble fibre content. More research is needed to determine whether the observed differences in *Ido1* expression correlates with microbial changes. An opportunity to investigate this will arise in the microbiota data of the study where soluble fibres are added to the HS diet.

The switch from a predominant *Bacteroidetes* population to *Firmicutes* in the gut has previously been linked to linked to DIO (Turnbaugh et al. 2008; Ley et al. 2006). However, recently it has been shown that the observed changes in the microbiota in mice on a high fat diet are largely due to the purification and lack of fibres in the diet (Dalby et al. 2017). In this study, they compared a chow diet with a HS diet and a HF diet, where starch and fat are exchanged and found, similar to our results presented in this chapter,

that the increase in *Firmicutes* is independent of the amount of fat but most likely related to the lack of soluble fibres in the diet. Here we additionally show that adding soluble fibre to a HS diet restores the amount of *Bacteroidetes* present in the ileum, with mostly inulin being very effective. Furthermore, genus profiles start moving towards profiles of starch fed animals when soluble fibres are added to the HS diet, indicating that the lack of soluble fibres is one of the main drivers for the differences in the microbiota between the chow and HS diet. However, the α-diversity score did not recover after adding any of the soluble fibres, most likely a combination of fibres is required (Lozupone et al. 2012).

A more in-depth look at differences in microbiota between these two diets showed four genera significantly higher in HS fed mice, of which *Allobaculum* and *Lactococcus* are capable of utilizing glucose for their metabolism (Herrmann et al. 2017) and *Mogibacteriaceae* have previously been shown to decrease with vegetable consumption (Kellingray et al. 2017). The only genera significantly lower on HS compared to Chow was an unidentified genus of the *S24-7* family. The *S24-7* family is known for its ability to ferment a wide range of carbohydrates, including fibres, of which the chow diet contains a large variety (Ormerod et al. 2016). In accordance with this, S24-7 increased after adding soluble fibres to the HS diet.

Metabolite profiles in the ileum are vastly different between the diets, with differences in both amino acids and carbohydrates present in both conventionally raised and broad-spectrum antibiotic-treated mice. Notably, the increase in amino acids in both the ileum and caecum of mice fed the HS diet, independent of the microbiota, could indicate malabsorption. This is emphasized by similar differences in the amino acid content found in the caecum of HS fed mice. While it has been shown in ileostomies that nitrogen loss from the ileum is common (Chacko and Cummings 1988), the difference between the diets are striking. Additionally, the differences in amino acids seem to be independent of the microbiota. Surprisingly, the HS diet provides less protein per kJoule (20 En% versus 27 En% in the chow diet) and energy intake is similar between the groups, however the differences in digestion between the diets remain unknown. In humans it has previously been shown that cereal fibres inhibit the increase in urinary nitrogen to creatine ratio, a biomarker for protein intake and digestion, in subjects on a high protein diet (Weickert et al. 2011). This indicates that cereal fibres interfere with the digestion and/or absorption of

proteins in the small intestine (Weickert and Pfeiffer 2018), however we find an almost opposite effect here, with less protein absorption on a low-fibre diet. More research will be needed to reveal the complex interaction between dietary fibres and protein uptake.

In line with the decreased health of the small intestine we observed a decrease in ileal villus length and an increase in intestinal permeability during long term (6 months) feeding of the HS diet. In chickens, adding dietary fibres to the diet has previously been shown to increase villus length in the ileum (De Maesschalck et al. 2015). This has been speculated to be due to butyrate formation from the present fibres; although our methods were not sensitive enough to determine butyrate in the ileum. However, in the caecum butyrate was decreased four-fold in HS fed animals. Sodium-butyrate supplementation is reported to increase ileal villus length in broiler chickens (Hu and Guo 2007) and butyrate has been shown to induce cell proliferation in the ileum (Canani et al. 2011). Most of the research so far in the effect of nutrition on small intestinal villus length is focussed on animal nutrition and feed-effectiveness. However, it would be interesting to test these effects in humans since the change in villus length correlated with changes in intestinal and liver health in our study. The decrease in butyrate throughout the intestine, as shown in the caecum, could also explain the increase in intestinal permeability. Butyrate has been shown to facilitate the assembly of tight junctions via the activation of AMP- activated protein kinase (AMPK) in the intestinal cells (Peng et al. 2009). Unfortunately, we were unable to identify the causal role of soluble fibres for intestinal permeability, as the length of time of soluble fibre feeding was too short to observe differences.

In the present study, no significant differences in weight gain were found between mice fed the chow or the HS diets. However, liver histology revealed damage to the hepatocyte architecture following HS feeding. These results were substantiated by our analysis of apoptosis via caspase 3 activity, triglyceride quantification and hepatic gene expression.

Taken together, with the initial signs of parenchymal damage observed in this mouse study on a HS diet, we believe investigating the differences between chow and HS is an interesting approach to look at the effect of dietary patterns on liver health disconnected from changes in body weight.

In conclusion, the HS diet is detrimental for gut and liver health, showing increased intestinal permeability and hepatic parenchymal damage and long-term even incidences

of hepatic carcinoma. Soluble fibres are in part able to counteract these effects, possibly via delaying starch digestion, lowering the glycaemic load, altering the microbiota and/or normalizing *Fgf15* expression (see chapter 4). However, more research is needed to identify both the long-term effects of soluble fibres on the small intestine–liver axis.

Chapter 6. Long term caloric restriction decreases bile acid levels in the ileum and a reduced expression of the enterokine Fgf15

Thesis Britt Blokker

Name study	Where	Performed by candidate?	Samples available
IDEAL study	WUR	No	Intestinal tissue Intestinal content Microarray data on liver tissue

6.1 Introduction

The health benefits of a calorie restricted diet (CR) while avoiding malnutrition are widely acknowledged. First, the lack of calories reduces the incidence of obesity and related metabolic diseases (Heilbronn et al. 2006). Secondly, CR has been shown to increase the life-span of a wide range of species, from yeast to mice and primates (Fontana, Partridge, and Longo 2010; Fontana and Partridge 2015).

Although it has become clear that bile acids act as metabolic hormone-like molecules (Kuipers, Bloks, and Groen 2014), we know very little about the role of bile acids and their metabolism in ageing during CR. Short term, it is known that plasma bile acid levels do not increase after CR in humans (Jahansouz et al. 2016; van Nierop et al. 2017). However, bile acids in the enterohepatic circulation increase after short term CR in mice in a dose-dependent manner (Z. D. Fu and Klaassen 2013). In this study, 30% and 40% CR for one month increased bile acids in enterohepatic circulation and their production via hepatic *Cyp7a1*, while a trend towards increased *Fgf15* expression was observed in the ileum (Z. D. Fu and Klaassen 2013). The authors also show that the increase in bile acids is independent of sirtuin 1 (*Sirt1*) (Z. D. Fu, Cui, and Klaassen 2015), a transcription factor that is activated in response to CR and is considered to mediate some of the health benefits provoked by CR (Cohen et al. 2004; Bordone and Guarente 2005).

An emerging area where CR is important in bile acid synthesis/physiology and metabolism is bariatric surgery (Albaugh et al. 2017). Bariatric surgery has been shown to increase circulating bile acids in mice (Ding et al. 2016), rats (Cummings et al. 2012) and humans (Steinert et al. 2013). Further, the important role of bile acid-sensing transcription factor FXR (Ryan et al. 2014) and of the G-protein coupled membrane receptor TGR5 (Ding et al. 2016) and their association with the beneficial effects of bariatric surgery have been elucidated.

On the other hand and in the case of shorter term CR, obesity has also been associated with an increase in circulating bile acids and elevated bile acid synthesis (Haeusler et al. 2016), and can be counteracted by three days of CR (Straniero et al. 2017). Additionally, circulating FGF19 levels are higher in lean than in obese people, but this could not be corrected by short term CR (Mráz et al. 2011).
Lastly, it has been known for a long time that ageing can influence bile acid metabolism. Hepatic conversion of cholesterol to bile acids has been shown to decrease in elderly populations (Einarsson et al. 1985) along with lower activity of CYP7A1, the rate limiting step in this conversion (Bertolotti et al. 1993). Moreover, bile acid secretion decreased in ageing rats, and this could be partly prevented by a 40% CR diet (Ferland et al. 1989).

Rusli *et al* (Rusli et al. 2017) performed a very interesting study in ageing mice where control fed mice were compared to 30% CR fed mice over 24 months. In collaboration with the group of Dr Wilma Steegenga (Wageningen University, NL), we were able to investigate bile acid metabolism and the microbiota from the small intestine in samples obtained from this study, providing a unique opportunity to investigate the effects of CR on bile acid metabolism during ageing. In this chapter, we show that bile acid metabolism is affected differentially during ageing in CR fed mice compared to control mice, showing an interesting decrease in both bile acids present in the ileum and the expression of signalling molecule *Fgf15* during ageing.

6.2 Materials and Methods

6.2.1 IDEAL study design

The IDEAL study has been performed at Wageningen University in accordance with the Dutch national guidelines. Our research group at the University of East Anglia was provided with small intestinal samples from this study. For IDEAL, male C57B6/J mice were housed individually at the age of 9 weeks and were randomly distributed into a control group (HS, n=89) and a CR (n=117) group. The control group received the AIN-93W diet ad libitum, a diet similar to the HS diet used in other chapters of this thesis (figure 6.1A). The CR mice received the AIN-93W-CR diet in portions containing 70 En% of the mean intake of the HS group supplemented with vitamins and minerals (figure 6.1B), provided daily at 4.30pm. Mice were sacrificed at 6, 12 and 24 months of age, providing the opportunity to investigate effects of CR during ageing. Body weight was measured every week (figure 6.1C) showing a decrease in body weight gain in CR fed mice. A more elaborate description of the study can be found in previous publications (Rusli et al. 2015; Rusli et al. 2017).

A

	UEA HS D12450H	IDEAL HS AIN-93W
Protein	20 En%	15 En%
Carbohydrate	70 En%	76 En%
Fat	10 En%	9 En%
Corn Starch (g)	452.2	495.7
Maltodextrin (g)	75	125
Sucrose (g)	172.8	100
Cellulose (g)	50	50

B

	HS AIN-93W	CR AIN-93W-CR
Energy (kcal/g)	3.85	3.77
Energy from fat (%)	9	10
Energy from protein (%)	15	15
Energy from carbohydrates (%)	76	75
Mineral mix AIN-93M (g%)	35	50
Vitamin mix AIN-93M (g%)	10	14
Choline bitartrate (g%)	2.5	3.5

C

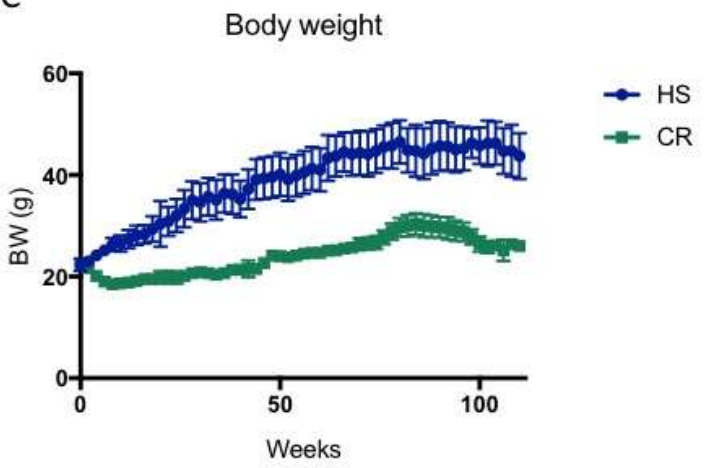

Figure 6.1 Diets and body weight. Here, the high starch (HS) diet used in the previous chapters of this thesis is compared with the HS diet used in the IDEAL ageing study (A). The caloric restriction diet used in the IDEAL study is based on the HS diet but compensated for both minerals and vitamins (B). Results for body weight were collected in Wageningen University and show the expected lower body weight in CR fed mice during ageing (C).

6.2.2 Samples analysed at UEA / Quadram Institute

As part of a collaboration with Dr. Wilma Steegenga at Wageningen University we received the small intestine of the mice fed the HS and the CR diet that were sacrificed after 6, 12 or 24 months. We received both the tissue of the small intestine and the small intestinal content.

6.2.3 Gene expression

RNA of liver and intestinal scraping samples was isolated. Samples were homogenised in 1 ml Trizol (Qiazol, Qiagen, UK) for 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France). After homogenization samples were transferred to a new Eppendorf tube and 200 µl Chloroform was added. Samples were centrifuged for 15 minutes at 12.000 RCF 4˚C and the clear layer was transferred to a new collection tube. Iso-propanol was added and after mixing, resting for 5-10 minutes and centrifugation for 10 minutes at 12.000 RCF 4˚C an RNA pellet was left. The pellet was washed in 85% ethanol and dissolved in 100 µl RNase Free water. RNA concentration was measured with a Nanodrop (Thermo Scientific, Wilmington, USA). cDNA was synthesized from 2 µg of RNA which was first treated with DNase (DNase kit, Invitrogen, 18068-015) and subsequently reverse transcription was performed. A quantitative polymerase chain reaction (Q-PCR) was performed using SYBR green master mix (Applied Biosystems, Thermo Fisher Scientific, UK) according to the producer's instructions. Reactions were performed using 384 wells (Applied Biosystems, Thermo Fisher Scientific, UK) using 5µL of SYBR green and primer mix (for a list of the primers used, see table 6.1) and 1.5µL of cDNA. The reaction was initialized at 50°C for 5 minutes and 95°C for 2 minutes, after which 40 cycles of denaturation (95°C 15 seconds) and annealing/extension (59°C 1 min) were performed. Afterwards, a melting curve was created and checked for a single product per gene. ΔCT values were calculated by subtracting the CT value of the housekeeping gene TBP from the gene CT value. ΔΔCT was calculated relative to control samples (Livak and Schmittgen 2001).

Table 6.1 Primers used for q-PCR measurements in small intestine.

Gene	Forward	Reverse
TBP (hk)	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT
GAPDH (hk)	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
FXR	GGCCTCTGGGTACCACTACA	AAGAAACATGGCCTCCACTG
OSTA	TTGTGATCAACCGCATTTGT	CTCCTCAAGCCTCCAGTGTC

ASBT	TGGAATGCAGAACACTCAGC	GCAAAGACGAGCTGGAAAAC
FABP6	CACCATTGGCAAAGAATGTG	AACTTGTCACCCACGACCTC
SHP	TCTGCAGGTCGTCCGACTATT	AGGCAGTGGCTGTGAGATGC
FGF15	CAGTCTTCCTCCGAGTAGCG	TGAAGACGATTGCCATCAAG

Selected samples went for microarray analysis. After RNA isolation as described before, samples were send to WUR for microarray analysis. At WUR RNA quality was assessed with a Bioanalyzer (Aligent 2100 Bioanalyzer, Santa Clara, USA), and microarrays were performed using the Mouse gene Chip 1.1 array from Affymetrix (Thermo Fisher Scientific, Santa Clara, USA). After normalization process, ~22 k genes were included in the data set. The limma R library was used to analyse the significant differential expression between the diets. Log2FC of 1.5 and a p-value of 0.05 were used as cut-off points and the rate discovery false for the p-value was calculated. Ingenuity Pathways Analysis (IPA) web-based software application was used to analyse, integrate and interpret the data

6.2.4 Bile acid measurements

As no bile acid measurements were performed in house at the time of arrival, we set up our own method in collaboration with the metabolomics unit of the Quadram Institute. A special thanks go to Mark Philo, who was a tremendous help in this process. Bile acids were measured in the scrapings and content of the three different parts of the small intestine, liver, colon faeces and serum. Here for, 50 mg (liver), 25 mg (intestine) or 50 µL (serum) of tissue was homogenised in 1 ml of cold 70% methanol for 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France). After centrifugation (5 min, 3000g, 4˚C) the supernatant was transferred to a new collection tube and MeOH content was removed by rotary evaporation (70 min, 50˚C). Volume was restored to 1ml by adding 5% MeOH. To clean up the samples they were loaded onto Waters OASIS PRIME HLB 1 30mg SPE cartridges, washed with 5% MeOH and eluted in 500 µl 100% MeOH.
Internal standards were added at the following time points: before homogenisation, before rotary evaporation, before loading onto cartridges, after elusion.

Cleaned-up extracts were analysed using HPLC – mass spectrometry operated in multiple reaction monitoring (MRM) mode.

Each sample (5 µl) was analysed using an Agilent 1260 binary HPLC couples to an AB sciex 4000 QTrap triple quadrupole mass spectrometer. HPLC was achieved using a binary gradient of solvent A (Water + 5mM Ammonium Ac + 0.012% Formic Acid) and solvent B (Methanol + 5 mM Ammonium Ac + 0.012% Formic Acid) at a constant flow rate of 600 µl/min. Separation was made using a Supelco Ascentis Express C18 150 x 4.6, 2.7µm column maintained at 40˚C. Injection was made at 50% B and held for 2 min, ramped to 95%B at 20 min and held until 24 minutes. The column equilibrated to initial conditions for 5 minutes.

The mass spectrometer was operated in electrospray negative mode with capillary voltage of -4500V at 550˚C. Instrument specific gas flow rates were 25ml/min curtain gas, GS1: 40ml/min and GS2: 50ml/min.

See appendix 2 for LC-MS conditions and mass fragmentation monitoring values. Quantification was applied using Analyst 1.6.2 software to integrate detected peak areas relative to the deuterated internal standards.

6.2.5 Microbiota analysis

6.2.5.1 microbial DNA isolation from SI content

The content of each part of the small intestine was gently pushed out of the intestine at the time of sacrifice and rinsed out using 200µ of PBS. 150µL of this was used to isolate microbial DNA from. In collaboration with the Hall lab (Quadram Institute, Norwich Research Park, UK), for DNA isolation we used the Qiagen DNA mini kit following an improved protocol with additional steps to ensure breakage of all bacterial samples kindly provided by Dr. M Lawson. Briefly, the samples were homogenised using glass beads for 4x 30 seconds at 6000 rpm in a Precellys®24 (Bertin Technologies, France) and heated to 95°C for 5 minutes in the middle. Additionally, samples were incubated with a lysis buffer containing 20mg/ml lysozyme after which the homogenising was repeated. Consequently, DNA was isolated using the Qiagen DNA mini kit following instruction from the producer. DNA quantity was assessed using both a nanodrop and a Qubit reader.

6.2.5.2 16S sequencing of the SI microbiota

A minimum of 50 ng of DNA was sent to the Earlham Institute (Norwich Research Park, UK) for quality assessment and 16S sequencing using the Illumina platform. Results were further analysed in collaboration with Wiktor Jurkoswki, PhD and Sarah Bastkoswki, PhD from the Earlham Institute.

The microbiome composition was established using QIIME version 1.9.0 (Caporaso et al. 2010). Paired reads were merged during the Quantitative Insights into Microbial Ecology Qiime 1.9.0 illumina workflow employed on a high performance computing cluster. The mapping file includes the forward and reverse linker primer sequence and was used for demultiplexing. Demultiplexing, quality filter and mapping file validation was carried out as described in the Qiime illumina workflow manual. Operational Taxonomic Units (OTUs) were picked within Qiime using the closed-reference OTU picking protocol with the RDP classifier by searching reads against the Greengenes database version gg_13_8 to the 97% level, and reads were aligned with PyNAST. A representative sequence tree was gained after clustering using Uclust version 1.2.22.

We calculated richness as defined through Chao1 (Chao A, (2000)) and diversity as defined by Shannon (Shannon C, (1948)) using the R package Phyloseq (version 1.18.0) (McMurdie P et al., (2013)). The Shapiro-Wilk test was used to assess normality and showed that the alpha diversity values are not normally distributed. We tested if diets had a significant impact on the mouse microbiomes alpha diversity using the Kruskal-Wallis test. A modified one way ANOVA as implemented in lmPerm (version 2.1.0, Wheeler R E, (2010)) was employed, which uses permutation tests instead of normal theory test, and was used to test for effects diet as covariates.

We used the fitZig function of the R package Metagenomeseq (Paulson et al. 2013) to establish significant differences in abundances of OTUs between groupings including covariates. We considered OTUs that were present in at least 25% of all samples.

6.2.6 Additional analysis

In addition to the measurement performed at UEA and Quadram, some measurements performed at Wageningen University were reanalysed. Here, microarray analysis was

performed from the livers of 4 mice per group (n total = 24), and our group extracted bile acid-related genes from this data set.

6.2.7 Statistics

Statistics have been performed using Graphpad Prism. The statistical tests used are indicated in each figure legend. Analysis of variance (ANOVA) provides a statistical test of whether the means of groups are equal. When groups were compared over time, a 2-way ANOVA with Bonferroni post-hoc test was used. When three or more groups were compared at a single time point, a 1-way ANOVA was used with Bonferroni post-hoc. Lastly, when two groups were compared at one time point, an unpaired t-test was used to test for significance. $p<0.05$ was considered overall as statistically significant and significance was indicated as ***= $p<0.001$ **= $p<0.01$ *= $p<0.05$.

6.3 Results

The IDEAL study has previously been shown to successfully prevent adverse health effects from ageing by CR (Rusli et al. 2017). Here, we aim to investigate the role of bile acids and bile acids metabolism during ageing and under CR.

6.3.1 Bile acids in the ileum initially increase in CR mice but decrease during ageing

Bile acids were measured in scrapings of the ileum and showed a different effect of ageing in the HS fed mice compared to CR mice. In the HS fed mice, no clear effect of ageing was found on the bile acids present in the ileum. However, during ageing in CR mice the total bile acids present in the ileal scrapings decreased, which led to a significantly different amount of total bile acids present in the HS versus CR mice in 24 months old mice (figure 6.2A). Additionally, the percentage of secondary bile acids present in the scrapings was lower in the CR fed mice, especially in old mice, indicating a difference in the microbiota (figure 6.2B). When the bile acids were analysed individually, the decrease in bile acids with ageing in the CR fed mice became very apparent (figure 6.2D). The bile acids that are primarily affected by ageing under CR conditions are CA, TCA, T-b-MCA and TDCA. No significant effects of ageing on individual bile acids in ileal scrapings were observed in HS fed animals (figure 6.2C).

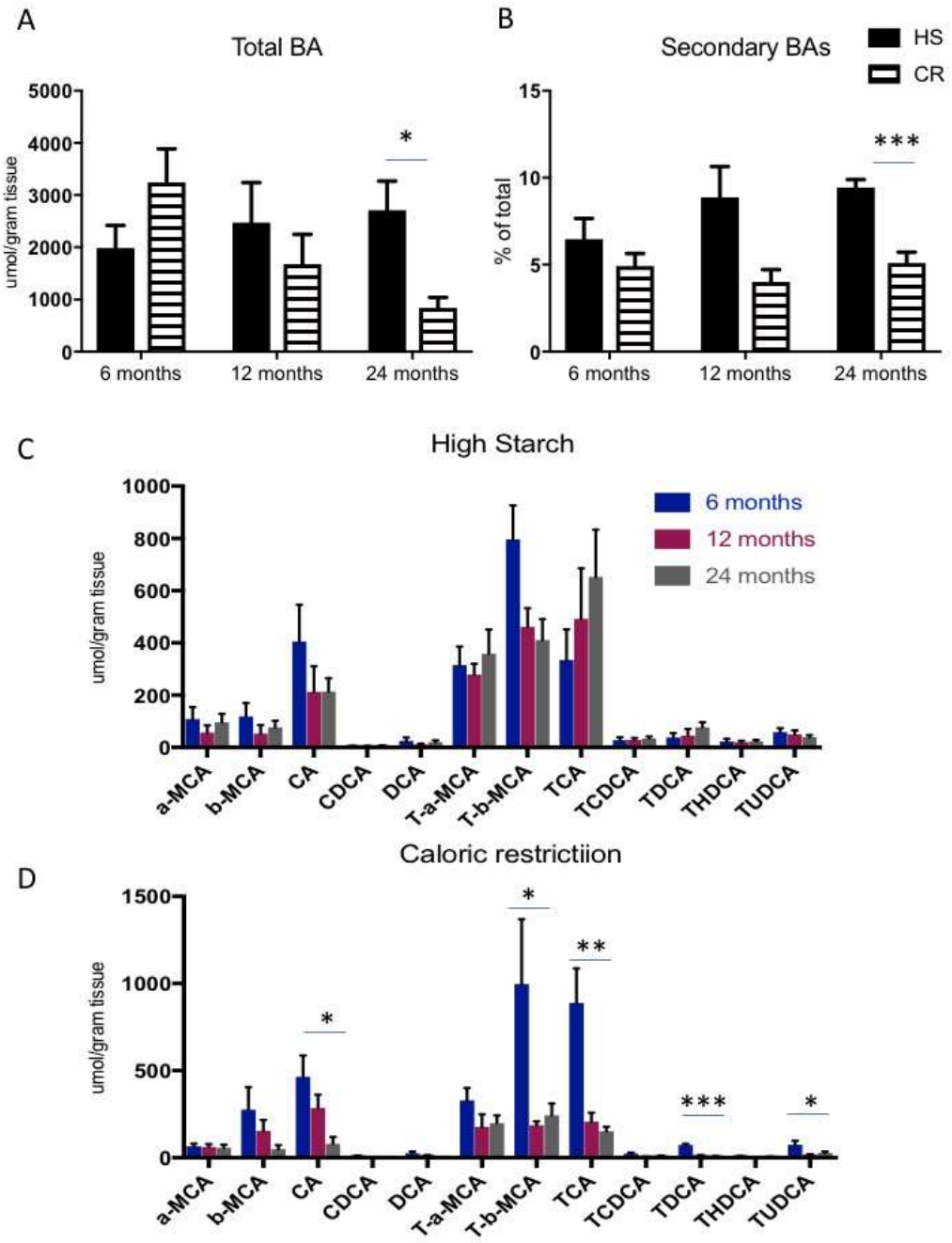

Figure 6.2 Bile acids first increase but during ageing decrease in CR fed mice. Bile acids were measured in ileal scrapings, with total bile acids show an initial increase and later decrease in CR fed animals compared to HS fed animals (A). There is a smaller portion of secondary bile acids in CR fed animals, which is significant after 24 months of feeding (B). Ageing does not significantly affect HS fed animals (C) but does significantly decrease multiple bile acids in CR fed mice (D). *Significance was tested using a 2-way ANOVA with Bonferroni post-hoc test in A and B and using a 1-way ANOVA with Bonferroni post-hoc test between all groups for C and D (*** = p<0.001, **= p<0.01, *= p<0.05) n=10 for all groups*

6.3.2 The expression of the signalling molecule Fgf15 is altered by ageing and CR

To investigate the consequences of the differences in bile acids in ageing in HS versus CR fed mice, genes involved in bile acid metabolism in the ileum were investigated. The signalling molecule Fgf15 increased during ageing in HS fed animals (HS 6 months versus 12 months $p<0.01$), while in CR animals *Fgf15* expression decreased with age (CR 6 months versus 12 months $p=0.04$). This opposite response observed with ageing leads to a difference in Fgf15 expression between the different diets at all time points, with an increased Fgf15 expression in adult CR fed mice and a lower expression in middle-aged and old CR fed mice (figure 6.3A). No difference was found in the expression of the gene responsible for bile acid uptake (*Asbt*, figure 6.3B), although activity measurements will be needed to determine if bile acid uptake is regulated post-transcriptionally between diets. Additionally, no changes were found in other FXR target genes involved in bile acid metabolism as shown by the expression of *Fxr* itself, *Fabp6* and *Osta* (figure 6.3C).

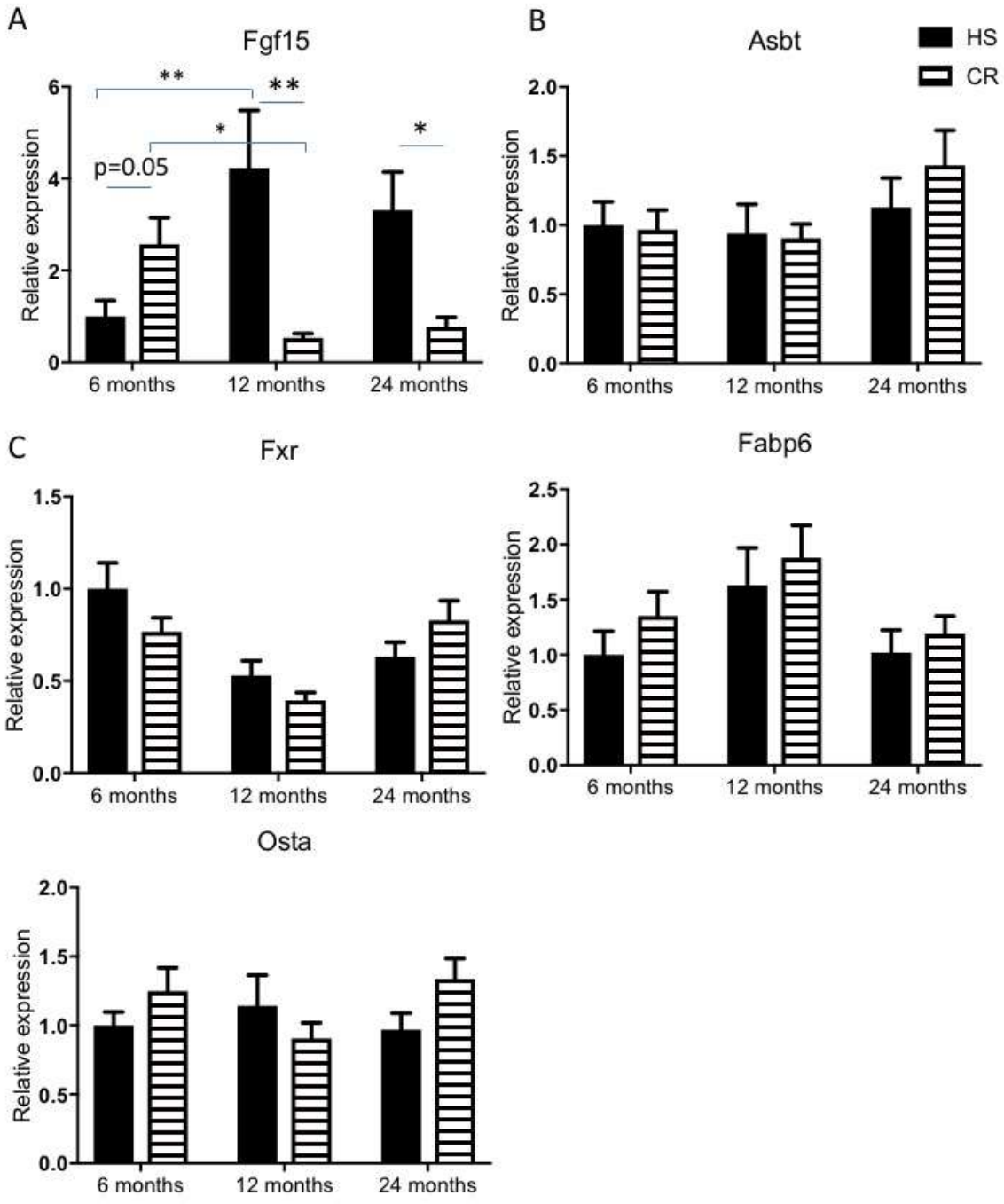

Figure 6.3 Gene expression of bile acid metabolism related genes in the ileum show Fgf15 being differentially regulated during ageing in HS compared to CR fed mice. Fgf15 expression is increased after 6 months of CR feeding but decreased after both 12 and 24 months of feeding a CR diet (A). Bile acid absorption is not altered at the transcriptional level during ageing on either diet (B) and FXR and FXR target genes also do not show differential regulation (C). *Significance was tested using a 2-way ANOVA with Bonferroni post-hoc test for both diet and time (**= p<0.01, *= p<0.05) n=10 for all groups*

6.3.3 CR influences *de novo* bile acid synthesis and transport in the liver

Since Fgf15 is known to inhibit hepatic *Cyp7a1*, the gene expression profiles in the liver were investigated for identifying genes involved in bile acid metabolism. *Cyp7a1* was found to increase in expression in CR animals, while no change was observed during ageing in control fed animals. Gene expression of *Cyp27a1*, responsible for alternative bile acid synthesis pathway, was found to be unaltered by both age and diet type (figure 6.4A).

When Fgf15 arrives at the hepatocytes via the portal vein, it binds to its receptor *Fgfr4*. Additionally, it has been shown that Klotho-β plays an important role in stabilizing FGFR4 after stimulation with Fgf15 (Triantis et al. 2010). Hence, expression of both *Fgfr4* and β-Klotho (*Klb*) was studied, with Fgfr4 expression increasing during ageing independent of the diet, and Klb expression being increased in adult and middle-aged but not old CR fed mice compared to control mice (figure 6.4B).

Lastly, gene expression of bile acid transporters in the liver were investigated. No changes were found for transporters at the basolateral or apical membrane, as shown here for *Ntcp* and *Mrp2* (figure 6.4C). However, transporters involved in alternative bile acid transport, responsible for transporting bile acids from the hepatocytes into the circulation (Halilbasic, Claudel, and Trauner 2013), were found to be influenced by both age and diet (figure 6.4D). *Mrp3*, the most highly expressed basolateral transporter, shows a downregulation in CR fed mice which is significant after 24 months. *Mrp4*, which is expressed in far lower amounts, shows an initial increase in CR fed animals, however an age-dependent decrease can be observed in these animals.

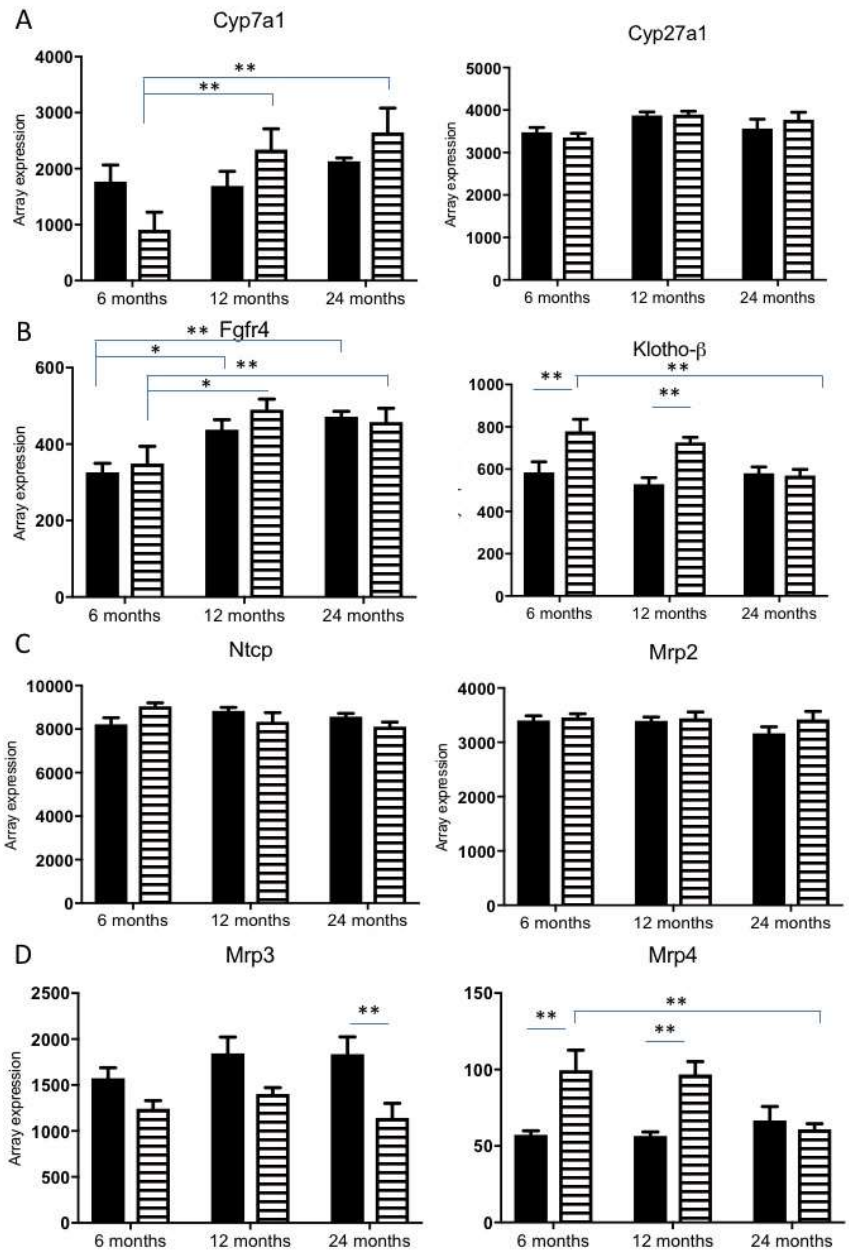

Figure 6.4 Hepatic Cyp7a1 and alternative bile acid transport is differentially regulated during ageing between the diets. Bile acid synthesis was investigated by gene expression of both Cyp7a1, showing an increase with ageing in CR mice, and alternative bile acid synthesis via Cyp27a1 expression showing no regulation (A). The FGF15 receptor dimer shows an age-dependent increase for Fgfr4 and a diet-dependent regulation for Klothoβ (B). Classical bile acid transporters on both the basolateral and apical membrane showed no regulation (C), while alternative bile acid transport was regulated by the dietary intervention (D). *Significance was tested using a 2-way ANOVA with Bonferroni post-hoc test for both diet and time (**= p<0.01, *= p<0.05) n=5 for all groups*

6.3.4 Microbiota profiles in the ileum are different in mice on the CR diet

To further investigate the role of CR in bile acid metabolism, we investigated the microbiota composition in the small intestine using 16S sequencing. Phylum profiles show a clear increase in *Actinobacteria* in adult and middle-aged CR fed mice and a slight decrease in *Bacteroidetes* (Figure 6.5A).

From the PCoA plots it becomes clear that the microbiota is significantly different between the control and CR fed mice at all measured timepoints ($p<0.01$ for all), however microbiota differences appear to be more pronounced after 6 and 12 months compared to 24 months (Figure 6.5B).

Alpha diversity was calculated using the Shannon diversity score. This showed similar diversity between the two diet groups and during ageing for the small intestinal microbiota, with the only difference being observed in 12 months old mice that showed a slight decrease in diversity in the CR group (Figure 6.5C).

Lastly, the microbiota was quantified by q-PCR against an E. Coli standard curve, showing no difference between diets, and a trend towards a decline in the number of bacteria present in the small intestine with age (Figure 6.5D).

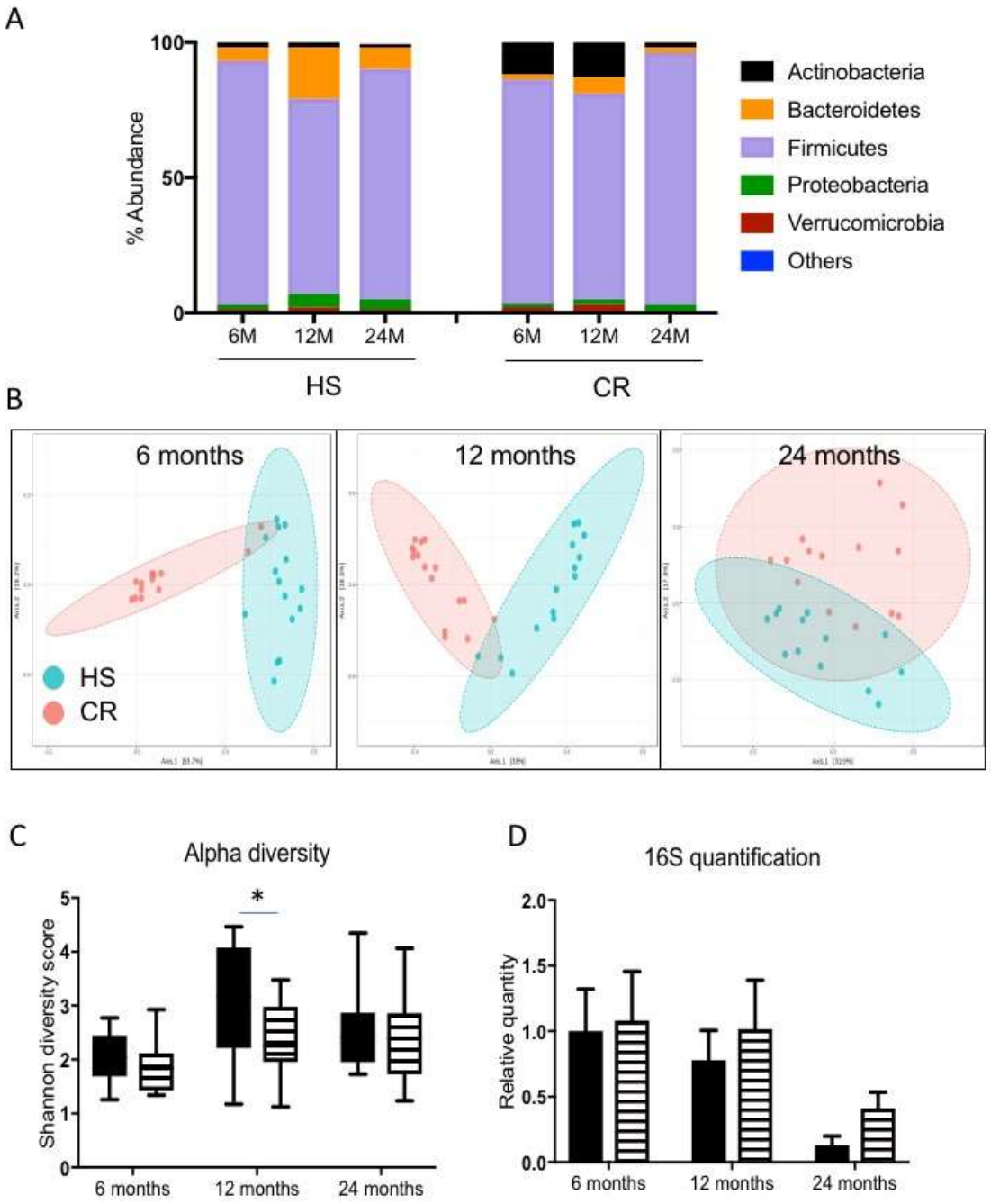

Figure 6.5 Microbiota composition in the ileum is different between HS and CR fed mice. Microbial DNA was isolated form the ileum and 16S DNA was sequenced using the Illumina platform. Phylum levels show differentially distinct profiles with more Actinobacteria in the CR fed mice (A). PCoA plots show the separation of the two diet groups which is most prominent at 6 and 12 months of age (B). Alpha diversity was calculated using the Shannon diversity score (C) and microbial DNA was quantified using Q-PCR (D). *Significance was tested using a 2-way ANOVA with Bonferroni post-hoc test for both diet and time (*= p<0.05) n≤5 for all groups*

6.3.5 More beneficial bacterial profiles in mice fed the CR diet for 6 and 12 months

The different genera present in the ileum of each mouse were studied to provide a more detailed observation into the small intestinal microbiota. The profiles of genera present are shown for each individual mouse and show clear differences between HS and CR fed mice (Figure 6.6A). A significant increase in *Lactobacillus* was observed in CR mice at all measured time points. In addition, at 6 and 12 months an increase in *Bifidobacterium* and a decrease in *Clostridiaceae* was observed in these mice. The genera *Allobaculum* was first found to be increased at 6 months of CR feeding, but after 12 months showed a slight decrease (Figure 6.6B). The increase in *Bifidobacterium* explains the previously observed increase in *Actinobacteria* (Figure 6.5A), as this genus belongs to the phylum of the *Actinobacteria* and a similar increase is observed.

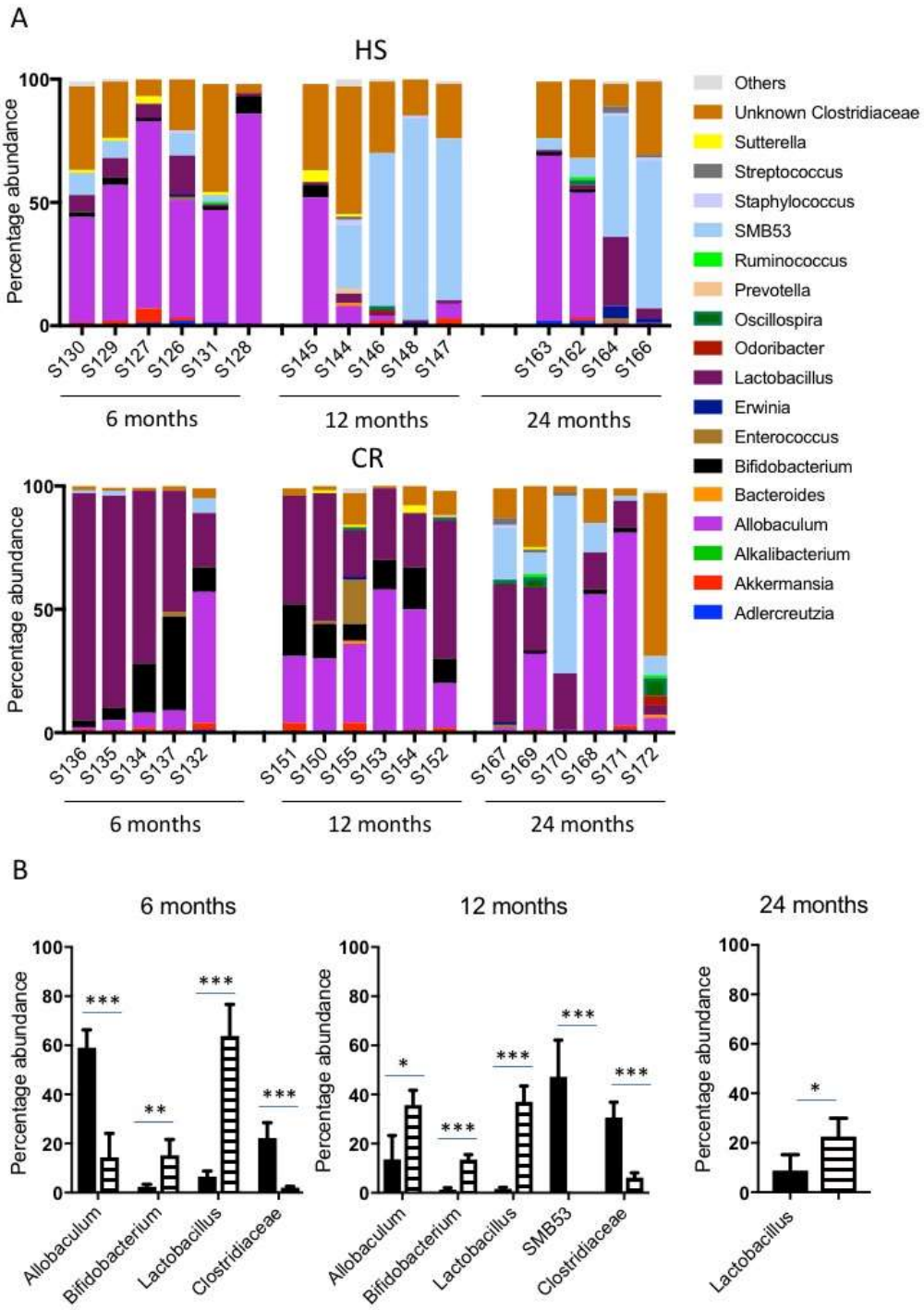

Figure 6.6 Multiple genera found to be differentially regulated in the ileum of CR mice. The genera present in the ileum are presented for each individual mouse, showing a clear difference between the diets (A). Significance was calculated using a Kruskal-Wallis test and after multiple measurement correction the significantly different genera between diets were plotted for each time point (B). *(*** = p<0.001, ** = p<0.01, * = p<0.05) n≤5 for all groups*

6.4 Discussion

We here show, for the first time, that very long-term CR (12 and 24 months) 1.5 to 2 fold decreases bile acids in the ileum and most likely via the transcription factor FXR decreases the expression of the important enterokine *Fgf15*.

As discussed prior, short-term CR has been shown to increase BAs in enterohepatic circulation (Fu and Klaassen 2013). In line with this finding, we show a slight increase in total BAs in the ileum after 6 months of CR, however ileal BAs decrease with ageing in CR mice. This ultimately leads to less ileal BAs in CR mice compared to HS fed mice. Previous ageing studies have focused on the liver and hepatic cholesterol excretion, showing that this decreases with age. Here, in HS fed mice we do not observe changes in ileal BAs. It should be noted that these bile acids are measured in the scrapings of the ileum, and after the content has been washed out, portraying bile acids present in both the enterocytes and the mucus layer. The colon of these mice have previously been investigated by the group of Dr Steegenga at Wageningen University and show that during CR bile acids are decreased in the colon in both adult and aged mice (Kok et al. 2018). This indicates a more efficient enterohepatic circulation of bile acids, since bile acids that have reached the colon have escaped reabsorption in the ileum. Although we do not observe changes in gene expression of the apical bile acid transporter *Asbt* in the ileum, its activity may be increased post-transcriptionally. Additionally, Straniero *et al* hypothesized that part of the mechanism in which bile acids escape reabsorption in the ileum may be related to the food content in the ileum (Straniero et al. 2017). In CR fed animals there is less food components that reaches the ileum, which could lead to more freely available bile acids and an increased efficiency of the enterohepatic circulation. Furthermore, CR mice were fed once a day with a restricted amount of food. Consequently, a fasting period was created each day for CR mice, as is usual in rodent CR models. Short fasting periods, even under iso-caloric conditions, have been shown to influence glucose metabolism and autophagy and mimic some health effects of CR (Martinez-Lopez et al. 2017). Iso-caloric restricted time feeding studies would be required to entangle the different effects of CR and short-term fasting on bile acid metabolism.

When bile acid metabolism was investigated in the ileum, *Fgf15* expression was found to be differentially regulated between the two diets. Correlating with the bile acid results for CR fed mice, *Fgf15* expression was increased in adult mice whereas it was decreased in ageing mice. An opposite ageing effect was found in HS fed mice. Not much is known about FGF15/19 during ageing though FGF15/19 levels have been proposed to be beneficial during ageing (Hao et al. 2013). An engineered, non-cancerogenic form of FGF19 was shown to decrease liver damage in aged mice (Alvarez-Sola et al. 2017). However, Fgf15 itself has been shown to provoke the incidence of hepatic carcinoma (Uriarte et al. 2015; Uriarte et al. 2013). In the current study, HS fed mice show extensive liver damage which was mostly prevented by CR, as published previously (Rusli et al. 2017).

Because of the known regulatory effects of Fgf15 on hepatic *Cyp7a1* expression (Kuipers, Bloks, and Groen 2014), liver bile acid metabolism was investigated. *Cyp7a1* gene expression did not change during ageing in HS fed animals. Previously, it has been shown that *Cyp7a1* activity decreases during ageing (Bertolotti et al. 1993), however this regulation might be post-transcriptional and therefore undetectable in our microarray results. In CR fed animals, *Cyp7a1* expression is first decreased and later increased which correlated with the regulation of *Fgf15* in the ileum.

Additionally, β-Klotho was found to be differentially regulated between the two diets, with higher expression in CR fed mice after 6 and 12 months of feeding. While the Klotho gene has been related to ageing and has been used to develop a short lifespan mouse model (Kuro-o et al. 1997), not much is known about the regulation of β-Klotho. β-Klotho-knockout mice show altered regulation of bile acid metabolism, but no effects on ageing phenotypes (Ito et al. 2005).

Furthermore, *Mrp3* expression was found to be increased in CR fed animals and this difference became more apparent during ageing. *Mrp3* is involved in alternative bile acid secretion from the liver into the bloodstream (Halilbasic, Claudel, and Trauner 2013). Unfortunately, we were not able to measure circulating bile acids due to a lack of samples. However, the increased expression of *Mrp3* indicated a possible increase in circulating bile acids, which can influence the regulation of the ubiquitously expressed G protein-

coupled bile acid receptor TGR5. TGR5 activation can regulate inflammation, obesity and diabetes (Duboc, Taché, and Hofmann 2014). More research will be needed to investigate the effects of very long-term CR on circulating bile acids and their role in regulating TGR5 activity in different organs.

Microbiota profiles of both diet groups show a large proportion of *Firmicutes*, which has traditionally been linked to the consumption of a HF diet. However, recently it has been shown that this is due to a lack of fibres in the diet. As discussed in chapter 5 of this thesis, the HS diet is a purified diet and does not contain any form of soluble fibre which explains the large proportion of *Firmicutes* (Dalby et al. 2017). The colonic microbiota composition of these mice has previously been analysed (Kok et al. 2018; van der Lugt et al. 2018). Although patterns are similar, there are some interesting differences between the small and large intestine. Firstly, *Bifidobacterium* is increased in presence in the colon of both adult and aged mice, while in the ileum only *Bifidobacterium* largely disappears at 24 months of age. In the colon *Bifidobacterium* spp are recognized for their health promoting properties because of their anti-inflammatory effects (O'Callaghan and van Sinderen 2016). In the ileum *Allobaculum*, a butyrate producer (Donohoe et al. 2011), is lower in abundance in adult CR mice, however it is higher in aged mice. In the colon, where most of the butyrate is produced, *Allobaculum* is found to be increased in aged CR mice. *Lactobacillus* are upregulated and *Clostridiaceae* downregulated in both the small intestine and colon at all timepoints.

The observed decrease in secondary bile acids indicates a decrease in BSH activity, a microbial enzyme present in the gut responsible for conversion of primary to secondary bile acids (Ridlon et al. 2014). BSH activity has been shown in bacteria belonging to the phyla *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Jones et al. 2008). Interestingly, both the *Lactobacillus* and the *Bifido* genera are known for their BSH activity. However, the microbiota is a complex mixture of bacteria, and the BSH enzyme is distributed across the major species (Ridlon et al. 2014). In germ-free mice inoculated with bacteria with and without BSH activity, BSH has been linked to a decrease in weight gain (Joyce et al. 2014). However, the decrease in secondary bile acids in our case is mostly due to a

decrease in deoxycholic acid (DCA), a secondary bile acid that has been linked to obesity and cancer in mice (Yoshimoto et al. 2013). We show here for the first time, that long term CR might decrease BSH activity in the small intestine. However, more research will be needed to confirm this and elucidate the health consequences of this mechanism.

BSH activity and nutritional regulation of BSH has previously been shown to increase FXR activity and thereby *Fgf15* expression (Sayin et al. 2013b; F. Li et al. 2013). In the current study, it is difficult to link the differences in the microbiota directly to the changes in bile acid metabolism since the microbial alterations proceed the changes in bile acid metabolism. No direct correlations were found between the microbial changes and the expression of *Fgf15* (data not shown), however this does not exclude a role for the microbiota. We hypothesize that CR influences bile acid metabolism via a complex interaction between hepatic, intestinal and microbial mechanism. More research will be needed to elucidate the molecular mechanism.

In conclusion, very long-term CR decreases the amount of bile acids in the ileum which leads to a reduction in *Fgf15* expression.

Chapter 7. General Discussion

Thesis Britt Blokker

7.1 Discussion

In this thesis, a novel regulation of the important gut hormone Fgf15 is described. To the best of our knowledge, this is the first time it has been shown that dietary changes are capable of regulating *Fgf15* expression independently of the microbiota. Starch digestion in the ileum, most likely via GlcNAcylation of the transcription factor FXR, induces the expression of Fgf15. Additionally, soluble fibres are capable of repressing its expression, hypothetically this might be caused by a reduction of starch hydrolysis and glucose uptake in the ileum. FGF15/19 has been linked to a large variety of health effects, including a decrease in weight gain in response to a HF diet (Fu et al. 2004) and the prevention of and/or a possible cure of NAFLD (Harrison et al. 2018). Purely for the sake of inducing *Fgf15* expression, a high starch low fibre diet would be ideal. However, this thesis also outlines the negative health effects of such a diet, showing dysregulations in the gut-liver axis that in our mouse model eventually leads to the development of hepatic carcinoma in a subset of mice. Lastly, a decreased consumption of this diet in a CR model, counteracts most of these negative health effects and additionally in the long term downregulates the expression of *Fgf15* in the ileum.

Speculative relevance of Fgf15 regulation by glucose

In this thesis, *Fgf15* expression was shown to be induced after ileal exposure to glucose. This could be biologically relevant because of the postprandial effects of FGF15/19 on glucose metabolism. Glucose, under normal physiological conditions, only reaches the ileum when the small intestine is overloaded with either starch or glucose. However, the Western style diet, which is becoming increasingly and widely adopted across the world, contains an excess of refined carbohydrates. Similarly to our findings, digestible starch has shown to reach the colon in mice fed a similar HS diet (Lange et al. 2015) indicating that overloading the capacity of duodenum and jejunum to digest starch and to resorb glucose leads to ileal starch exposure. Potthoff *et al* showed that Fgf15 acts subsequently to insulin to decrease blood glucose levels by regulating the hepatic CREB-PGC1α signalling cascade (Potthoff et al. 2011), a pathway that we also show to be affected by the glucose-induced *Fgf15* expression (appendix 3). Additionally, in humans circulating FGF19 has been shown to increase after the consumption of glucose (Morton et al. 2014;

Matysik et al. 2011) Thus, the induction of FGF15/19 due to glucose reaching the ileum could potentially help the body deal with a high postprandial glucose load.

Is starch or sucrose responsible for the increase in *Fgf15*?

In chapter 3, ileal glucose absorption was shown to induce Fgf15 expression, presumably from the digestion of starch. However, the HS diet also contains 3-fold more sucrose compared to the chow diet. Nonetheless, the starch content of the diet is held responsible for the increase in Fgf15 expression for two reasons. Firstly, in chapter 5, the gene involved in the conversion of fructose to glucose, *Fbp1*, is shown to be upregulated in the duodenal part of the small intestine. This indicates that fructose, and hence the sucrose, is taken up in the proximal and middle part of the small intestine. Secondly, when the improved HS diets were designed, the HS low sucrose diet one diet was identical to the original HS diet except for its sucrose content. Thirdly, a high fat purified diet with the same sucrose concentration but lower starch content led a similar Fgf15 expression as chow diet. In chapter 4, this low sucrose HS diet induce the expression of FGF15 almost 15-fold, indicating that the starch but not sucrose was responsible for the induction of FGF15.

Possible alternative modes of regulation of FGF15/19

Although in this thesis *Fgf15* expression seems to be increased by activation of the transcription factor FXR, other factors could also influence its regulation. As mentioned in section 1.5.2 of the introduction, FGF15/19 expression is regulated through multiple pathways. One major pathway involved in the regulation of FGF15/19 is via vitamin A. This activation pathway is dependent of FXR as shown in FXR knockout mice (Schmidt et al. 2010). In chapter 5 of this thesis, we discussed that the HS diet leads to a disturbance of vitamin A uptake in the small intestine. This was also found by Goverse *et al* demonstrating that vitamin A absorption was dependent on SCFA production in the small intestine (Goverse et al. 2017). This is partly confirmed in chapter 5 of this thesis, as we demonstrated that adding soluble fibres to the diets restores *Bco1* expression in the jejunum, a marker for vitamin A metabolism. Additionally, after treatment with antibiotics, *Bco1* was upregulated in both the chow and HS fed animals (data not shown).

It is this dependency on the commensal microbiota and possibly their metabolites which uncouples vitamin A metabolism from *Fgf15* expression in our models. After depletion of the microbiota by broad-spectrum antibiotics, *Fgf15* expression was still differentially regulated between diets and could additionally continue to be downregulated by adding the highly fermentable soluble fibre FOS in combination with a broad-spectrum antibiotics treatment.

Furthermore, Fgf15 expression can be regulated indirectly via the transcription factor PPARα (Zhou et al. 2014), or Diet1 (Vergnes et al. 2013) and by circadian rhythms under the control of KLF15 (Han et al. 2015). While these factors may play a role in the regulation of Fgf15 in our models, the results suggest that FXR is the most prominent regulator. The potential effects of circadian rhythms were avoided as much as possible by sacrificing all mice in the morning, between 8am and 12pm. Furthermore, no difference was found in the gene expression of *Diet1* in the ileum of chow and HS fed mice as tested by Q-PCR (data not shown). Lastly, while the expression of PPARα target genes was found to be downregulated by the HS diet in particular in the proximal and middle part of the SI (chapter 5), no obvious improvements of PPARα activity, as measured by target gene expression, could be found when fibres were added to the HS diet. In conclusion, although we cannot exclude other possible factors, here we demonstrate that in response to the HS diet the regulation of Fgf15 is most likely coordinated by FXR.

Health effects of dietary fibres: new mechanism?

As discussed in chapter 4 and 5 of this thesis, consumption of dietary fibres has been indisputably linked to improved health in humans (Kendall, Esfahani, and Jenkins 2010) for numerous reasons (see introduction). Here we show a potential new mechanism by which dietary fibres increase metabolic health, and additionally the less well understood role of dietary fibres for the small intestine is uncovered. In chapter 4, soluble fibres (FOS, inulin, pectin and fibersol) were shown to decrease the expression of *Fgf15*. This decrease could influence the conversion of cholesterol to bile acids via increased hepatic *Cyp7a1* expression, and may contribute to the known cholesterol-lowering properties of soluble fibres (Brown et al. 1999; Han et al. 2015b; Gunness et al. 2016). *Cyp7a1* overexpression has previously been shown to reduce blood cholesterol levels (Li et al. 2011). This finding

could potentially also be of importance for the treatment of bile acid diarrhoea (BAD), since under these conditions increasing FGF15/19 is desirable (Walters 2014). Although more research will be needed to determine the relative significance in humans, a low fibre, high starch diet could potentially benefit BAD patients. Current dietary recommendations for BAD patients are to follow a low-fat diet, while no consistent recommendations on fibre intake are currently available (Jackson et al. 2017).

Despite this being a very exciting new mechanism by which soluble fibres potentially lower cholesterol levels, it remains partly unclear how soluble fibres influence the expression of *Fgf15*. In chapter 4, we hypothesized that soluble fibres delay the uptake of glucose, possibly through the delay of the digestion of poly-, oligo- and disaccharides by blocking the activity of numerous α-glucosidases. While more research is needed to confirm this (see future prospects), others have suggested similar mechanisms for inulin (Neyrinck et al. 2016). Although finding this mechanism was not our aim, more research on this subject may elucidate a new role for soluble fibres in controlling blood glucose levels. Additionally, the delay in digestible carbohydrate uptake could influence the microbiota in both the small and large intestine (Lange et al. 2015), leading to additional health effects.

Although finding one of the potential mechanism of how soluble fibres decrease cholesterol levels is exciting, this might not influence current practice for the healthy population. In the UK, the current dietary guidelines advise 30 grams of fibres (SACN 2015) however the majority of the population does not reach this recommended amount. The results in this thesis do not show reason to change these recommendations, however might emphasize the importance of the consumption of dietary fibres, in particular soluble fibres.

The HS diet as a research model

Overall in this thesis, different advantages and disadvantages of a semi-purified HS diet have been revealed. Interestingly, the HS diet does not change the body weight of the mice compared to mice fed a standard chow diet. This potentially makes the HS diet an extremely interesting research model to investigate the role of nutrition on gut-liver health without the interference of increased weight gain and development of obesity. Obesity has

been linked to elevated levels of C-reactive protein in a meta-analysis of 51 cross-sectional studies (Choi, Joseph, and Pilote 2013), indicating systemic low-grade inflammation. This has been linked to the changes in both gut microbiota composition and intestinal permeability (Cox, West, and Cripps 2015). However, low-grade systemic inflammation during obesity is also influenced by inflammation of expanding adipose tissue (Maachi et al. 2004). The HS diet provides an opportunity to investigate the health impact (e.g. effects on liver health) of an altered microbiota as well as long-term and prolonged intestinal permeability without the interference of the expanding adipose tissue.

While on one hand this is mechanistically very interesting, it might also be relevant for human biology. The presence of NAFLD or NASH in the absence of an increased BMI has been named "lean NASH" and has been encountered in both Asia and the Western world, however its prevalence remains unclear (Das and Chowdhury 2013). While some genetic mouse models for NAFLD, like Srebp-1c KO mice, also develop NAFLD without developing obesity (Takahashi, Soejima, and Fukusato 2012), these models might be less suited for nutritional research. As opposed to pharmacological research, nutrition provokes modest effects, and is often more suited for prevention. A mild model that over time leads to the development of NAFLD without the interference of obesity, like the HS diet, might be ideal for nutritional research. Adding bioactive food components or replacing ingredients from the diets to prevent or even improve the detrimental liver phenotypes could potentially have a large contribution to our knowledge on the biological effects of nutritional components and would also allow evidence-based strategies to reformulate ultra-processed convenience foods that are frequently consumed by the UK population.

From this thesis, information about the HS diet phenotype can be drawn (see table 7.1). In summary, the HS diet has been found to increase the incidence of both NAFLD and liver cancer in mouse models. Adding soluble fibres to the diet can partly prevent the development of early stage NAFLD but longer experiments are needed to investigate its beneficial effects on hepatic cell proliferation and the development of liver cancer. Since the development of hepatic carcinoma has previously been linked to increased FGF15/19 signalling (Uriarte et al. 2015; Alvarez-Sola, Uriarte, Latasa, Urtasun, et al. 2017), and

soluble fibres decrease the expression of *Fgf15* (chapter 4), a decrease in hepatic carcinoma development is hypothesized on the long term. Furthermore, liver phenotypes have been shown to significantly improve when the HS diet is consumed in a caloric restricted way which is shown in CR mice (Rusli et al. 2017).

When looking at the small intestinal microbiota the switch between phyla from predominantly *Bacteroidetes* in chow fed mice to mostly *Firmicutes* in HS fed mice is well-defined, and has recently been shown to also be true for the colonic faecal microbiota by others (Dalby et al. 2017). In this thesis it is shown that the small intestinal *Bacteroidetes*: *Firmicutes* ratio can be increased by adding soluble fibres to the HS diet, but not solely by limiting the intake of calories of this diet.

In line with this finding, vitamin A metabolism seems to improve in response to the addition of soluble fibres (chapter 5). This is in agreement with previous findings that vitamin A metabolism is dependent on the production of SCFA in the small intestine (Goverse et al. 2017). The effects of CR on vitamin A metabolism remain unknown and require further investigation.

Gene expression of many important metabolic and immune-related pathways in the small intestine are also differential regulated between chow and HS fed mice. For example, AhR signalling is downregulated in HS fed mice. Activity of the AhR, as measured by the expression of its target gene *Cyp1a1*, could not be increased by either adding soluble fibres to the diet or decreasing caloric intake. Furthermore, *Glut2* expression (among other glucose uptake genes) was increased over the long term in the ileum of HS fed mice. Short term Glut2 was even further increased by adding soluble fibres, while CR normalized its expression. Lastly, as discussed in detail throughout this thesis, the HS diet increased the expression of *Fgf15* in the ileum. This could be decreased by adding soluble fibres to the diet and additionally in the long term via CR.

Table 7.1 Summary of the effects of the HS diet on obesity and the gut-liver axis. Additionally, the effects of adding soluble fibres or limiting the caloric intake on the HS diet are summarized.

	Chow	HS	HS-Fibre	HS-CR
Obesity	→	→	→	↘
Liver steatosis/NASH	→	↑	→	↘
Liver Cancer	↓	↑	?	↓
Bacteroidetes/Firmicutes	↑	↓	↓	↓
SI Vitamin A metabolism	→	↓	→	?
AhR signaling	↑	↓	↓	↓
Ileal Glut2	→	↑	↑	→
Ileal Fgf15	→	↑	→	> 6M ↓

Using mice as a model for humans in bile acids research

Current knowledge on bile acids in enterohepatic circulation is largely extrapolated from animal experiments, with rodent studies being the most prominent (Rudling 2016). However, there are several large differences between bile acid metabolism of humans and rodents which are important to recognize. One important difference is that in rodents bile acids are hydroxylated at the 6-β position to form muricholic acids (MCAs), which is unique to rodents (Takahashi et al. 2016). It has been discussed previously, that in germ-free conditions, the bile acid T-β-MCA reaches the ileum and antagonizes FXR to decrease Fgf15 expression (Sayin et al. 2013b). However, since no MCAs are present in humans, it is not known how this mechanism may be relative in a human context. Importantly, antibiotic treatment has also shown to decrease circulating FGF19 in humans (Vrieze et al. 2014), possibly via a very similar mechanism involving UDCA (Mueller et al. 2015).

Another important different lies in the fact that mice are one of several species that are known to eat their own faeces, a behaviour called coprophagy (Negro et al. 2002; Torrallardona, Harris, and Fuller 1996). Coprophagy can have major effects on the metabolism of different compounds, among which are bile acids. Bile acids that escape

reabsorption in the ileum are exposed to the large number of microbiota present in the caecum and colon. This leads to both deconjugation and the formation of secondary bile acids. In the case of coprophagy, these bile acids re-enter the circulation via the small intestine. Groen *et al* have showed that in the case of cholic acid feeding, the observed increase in circulating DCA is due to coprophagy. Importantly, they also show that in wild type mice on a chow diet, coprophagy has very little effect on circulating bile acids (Annemiek Groen, Cindy Kunne, and Elferink 2006). Coprophagy can be avoided by housing mice in metabolic cages, where due to a metal grate floor mouse are unable to eat faeces. Unfortunately, under UK regulations and due to limitations in the animal licenses held by our research group, we were unable to perform experiments with mice in metabolic cages.

Other limitations

We, in chapter 5 of this thesis, and others (Pellizzon and Ricci 2018) have discussed the multitude of differences between chow and semi-purified diets, however, in this thesis these diets are compared. To circumvent this, we attempted to isolate the effects of the separate ingredients. For example, by inhibiting starch digestion by adding acarbose in chapter 3 and by adding soluble fibres to the semi-purified diets in chapter 4 and 5.

7.2 Future prospects

Human relevance

In this thesis, dietary regulation of Fgf15 expression is shown in a mouse model and although there are indications that glucose is also capable of regulating FGF19 in humans (Morton et al. 2014), more research is required to determine its relevance in humans. Circulating FGF19 in humans can be measured via sandwich enzyme-linked immunosorbent assays (ELISA) from blood samples. A small human study where blood samples are taken postprandially after the consumption of a high starch, low fibre meal could confirm the rise of FGF19. As a control, a meal with a lower glycaemic index, replacing the simple carbohydrates by fat, could be used.

Furthermore, a slightly longer study, where participants receive the diets for a week, would be advisable to pinpoint the effects of soluble fibres on circulating FGF19 in humans. A low fibre diet containing substantial amount of digestible starch (for example in the form of white rice) should be compared with a diet containing more sources of soluble fibres (for example supplemented with inulin).

Because of the low amounts of sample needed to confirm the mechanism there might be an opportunity to use samples from existing human intervention studies, however it should be considered that blood samples need to be taken in the non-fasted state. Postprandial FGF19 levels, compared to fasted levels, have previously been shown to be more effective in the case of diagnosing BAD (Borup et al. 2015). Importantly, Matysik *et al* (Matysik et al. 2011) hypothesize that FGF19 found in the circulation is merely the "tip of the iceberg" of the FGF19 produced in the ileum, assuming a rapid and efficient transport from the ileum to the liver. It is important to emphasize that circulating FGF19 most have escaped the gut liver axis, as usually the FGF19 produced in the ileum is directly transported to the liver via the portal vein. So even though these studies are relatively simple and realistic they might not give all the needed answers.

In order to really investigate how dietary patterns influence FGF19 expression in humans, ileal tissue samples are needed. Although more difficult to obtain, new techniques are being developed in the form of pills that measure in vivo in the human gastro-intestinal tract (Kalantar-Zadeh et al. 2018). Currently, the technique is optimised for measurements

as pH, however it is not unimaginable that in the future similar pills wills be able to take small samples of intestinal tissue. Since only small amounts of tissue are needed for gene expression analysis this could in the future be used to study the expression of FGF19. First, a confirmation of the increase of FGF19 expression after a high starch and/or glucose diet as opposed to a high fat diet should be confirmed (Morton et al. 2014), after which the effect of the addition of soluble fibres to the high starch diets could be investigated.

GlcNAcylation of FXR

In chapter 4 it is hypothesized that Fgf15 expression is increased in the ileum of HS fed animals because of GlcNAcylation of FXR. To confirm this, both *in vitro* or *in vivo* experiments should be performed. The main challenge with *in vitro* experiments is the lack of relevant cell lines, however this could be circumvented by the use of primary cells. Primary cells isolation of the ileum could be performed as described (Graves et al. 2014), from mice on a chow background. Consequently, the primary cells should be incubated in high and low glucose medium in the presence or absence of the bile acid CDCA. CDCA is a very potent FXR agonist (Bramlett, Yao, and Burris 2000). If GlcNAcylation of FXR leads to an increase in *Fgf15* expression, *Fgf15* expression should be significantly increased in the CDCA stimulated high glucose group compared to the CDCA stimulated low glucose group. Because GlcNAcylation only stabilised the protein does not activate it, no difference in expression of *Fgf15* is expected in the non-stimulated groups.
Additionally, *in vivo* experiments can confirm an overall increase is GlcNAcylation. Based on a previous publication where hepatic FXR has been shown to be glycosylated (Berrabah et al. 2014), this can be investigated by using a combination of both western blotting and immunoprecipitation of using sWGA-agarose beads. *In vivo* more GlcNAcylation should be expected in the ileum of mice fed the HS diet.

Effects of dietary fibres on the gut-liver axis

Based on the result presented in chapter 4 and 5 of this thesis, the soluble fibres inulin, pectin and fibersol can counteract some of the adverse health effects provoked by the HS diet. In order to further explore the effects that the fibres have on the gut-liver axis, longer feeding experiments with these diets would be recommendable. As discussed, the HS diet

is a mild model, however it leads to development of relatively extreme liver phenotypes and increased intestinal permeability over time. To investigate if soluble fibres can prevent this, 6 months of feeding these diets would be advisable. After 6 months, outcome measurements should include liver phenotyping via histology, intestinal permeability measurements via FITC dextran, bile acids in the ileum and serum, microbiota determination via 16S sequencing and gene expression in both the intestine and liver.

The impact of dietary soluble fibres on small intestinal glucose metabolism and uptake

As discussed previously in this chapter, during this thesis we encountered a potentially very interesting new way of action for soluble fibres. We, in line with others (Neyrinck et al. 2016), show that soluble fibres potentially inhibit α-glucosidases relevant e.g. for starch and sucrose hydrolysis in the small intestine. However, further research is required to elucidate this mechanism in detail. So far, we have presented gene expression data to support this theory, however activity measurements of the α-glucosidases along the course of the small intestine (duodenum, jejunum and ileum) would provide more information. A ex-vivo method for activity measurements in the mucosa has been described by Dahlqvist (Dahlqvist 1968) and has been improved by Oku *et al* (Oku et al. 2014). As a positive control Acarbose should be included into the protocol of the experiment. Additionally, broad-spectrum metabolomics on all parts of the small intestine will give a better understanding of the digestion of carbohydrates from stomach to colon.

Additionally, *in vitro* models could help untangling the mechanism. Due to the previously discussed lack of commercially available small intestinal cells line, primary cells culture would be advisable. Again using Acarbose as a positive control for the inhibition of α-glucosidases, the amount of glucose produced from oligo- and disaccharides in the presence or absence of soluble fibres could potentially be used as the outcome measurement, as described here (Neyrinck et al. 2016).

Vitamin A metabolism in the small intestine

In chapter 5 of this thesis vitamin A dysregulation in response to the HS diet is discussed. Others have shown that for optimal absorption of vitamin A, SCFA are needed in the small intestine (Goverse et al. 2017). While the results presented in this thesis support the need

of the small intestinal microbiota, further research might reveal this mechanism in more detail. So far, we have shown that in the HS diet vitamin A metabolism is dysregulated in the small intestine and that this can be at least party be prevented by adding soluble fibres to the HS diet, as shown by the expression of *Bco1*. Additionally, antibiotics treatment further dysregulated the uptake of vitamin A. However, the production of SCFA in the small intestine is relatively low so potentially other metabolites produced by the microbiota maybe also involved. Broad-spectrum metabolomics on the duodenal and jejunal content of mice fed a chow, HS and HS with added fibre diet could provide some candidates. Furthermore, a microbial transplant from HS to HS with added fibres mice and vice versa could provide mechanistical prove that the microbiota is responsible for the regulation of vitamin A metabolism in the small intestine.

Bile acid metabolism under long-term CR

While we were very grateful for the opportunity to study bile acid metabolism in the long-term CR study IDEAL (chapter 6), some of the mechanisms by which CR influences bile acid metabolism and the bile acid pool size remain uncertain. If at all possible, measuring bile acid in the serum of these animals would add valuable information. Measuring circulating bile acids will provide information about how the change in bile acids is affecting other organs, outside of the liver and intestine.

Furthermore, it would be extremely interesting to investigate the role of the microbiota in the changes in bile acid metabolism. However, due to the long-term studies to simulate ageing to observe the effects, eradicating the microbiota by broad-spectrum antibiotics might not be possible. An alternative could be to perform an inoculation experiment with the microbiota of mice that have been on the HS and CR diet for at least 12 months. Inoculation can take place in antibiotic treated mice, as described here (Ellekilde et al. 2015). Although in chapter 6 it is discussed that the microbiota changes precede the changes in bile acids, this is only for the microbiota part that can be characterize by 16S sequencing. During inoculation the entire microbiota is transplanted, including metabolites produced by the microbiota, fungi and archaea. Investigating the effects of transplanting microbiota into a healthy host mouse after antibiotic depletion of the intrinsic

microbiota, could provide us with additional mechanistic information about the ways in which CR influences the gut-liver axis.

List of communications and courses

Oral communications:

Dietary fibres regulate bile acid metabolism independent of the microbiota by supressing the production of the signalling molecule Fgf15 in the ileum
Britt Blokker, Elí Gámez, Francesca Manocchio, Sarah Bastkowski, Mark Philo, Gwenaelle Le Gall, Wiktor Jurkowski, David Vauzour, Naiara Beraza, Michael Müller
Annual NuGO Conference, Varna, Bulgaria *August 2017*

Dietary fibres regulate bile acid metabolism independent of the microbiota by supressing the production of the signalling molecule Fgf15 in the ileum
Britt Blokker, Elí Gámez, Francesca Manocchio, Sarah Bastkowski, Mark Philo, Gwenaelle Le Gall, Wiktor Jurkowski, David Vauzour, Naiara Beraza, Michael Müller
Rank Prize Symposium and Carbohydrates and Health, Grasmere, UK *August 2017*
→ Prize for best oral communication

Role of the dietary composition on bile acid metabolism and its impact for the gut-liver axis
Britt Blokker, David Vauzour, Mark Philo, Gwenaelle Le Gall, Naiara Beraza, Michael Müller
European Club for Liver Cell Biology Meeting, Ascot, UK *October 2016*

Dietary regulation of bile acid metabolism
Britt Blokker, David Vauzour, Mark Philo, Gwenaelle Le Gall, Naiara Beraza, Michael Müller
Annual Student Conference, UEA Medical School, Norwich, UK *September 2015*

Poster communication:

- Student Science Showcase, Quadram Institute, *September 2017*
  - ➔ Prize for best communicated poster
- Student Science Showcase, Quadram Institute, *September 2016*

Attended courses:

- **Home office course module 1 and 4**
  12/01/2015 and 15/01/2015 at Cambridge University
  Aim: To acquire a licence to work with laboratory animals in the UK.
- **Competency training by the NTCO**
  01/2015 and 02/2105 at the Disease Modelling Unit at the UEA
  Aim: To become competent to perform procedures on laboratory animals.
- **MOLECULAR NUTRITION & REGULATION OF CARDIOMETABOLIC HEALTH**
  June 2015 at University College Dublin, Ireland
  Aim: To learn about the molecular mechanisms underlying the effects of nutrition on cardiometabolic health
- **Knowledge Exchange Trip Wageningen University**
  August 2015 Wageningen, The Netherlands
  Aim: Visiting the facilities of Wageningen University, attending presentation by PhD students from both WUR and the Quadram Institute. Presenting my own work.
- **Nutrigenomics studies in humans: from epidemiology to intervention**
  September 2015 at Universidad de Barcelona, Spain
  Aim: To learn about how nutrigenomics can be implemented in human intervention studies
- **Connecting Nutrition and Health**
  January 2017 Earlham Institute
  Aim: To learn about the integration of omics data, with a particular focus in the fields of health and nutrition as well as environmental factors influencing health.
- **Knowledge Exchange Trip Dublin**
  August 2016 Dublin, Ireland

Aim: Visiting the facilities of Dublin University and Trinity College Dublin, attending presentation by PhD students from both WUR and the Quadram Institute. Presenting my own work and being the main organizer of this trip.

Public Engagement:

- **Big Bang Fair**
  March 2015 in Birmingham
  Activity: Manning the IFR stand at the Big Bang Fair on both Friday and Saturday. Friday was a school day and Saturday a family day. During both days we played food and health related games with the children to try to interest them in nutrition and health.
- **Forum Science day**
  May 2015 in Norwich
  Activity: Playing similar games with the children as during the Big Bang Fair to interest them in the health effects of food
- **PubHD**
  February 2016, the Cellar House (pub), Norwich
  Activity: Giving a 10 minute presentation about my PhD to a lay audience and answering questions
- **IFR student Forum – Events officer**
  Academic year 2016-2017
  Activity: Organising events for the PhD student (and sometimes staff) of the IFR. Examples of the organised events are a games night for charity and the student Christmas party.
- **Norwich Science Festival**
  October 2017, Forum, Norwich
  Activity: 30 minutes oral presentation to the general public with discussion afterwards.

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Luckily, there were more PhD students at the Quadram Institute, so we could struggle together. We have known some good times in the student forum and the Christmas parties, BBQs and international trips gave the needed support and some time to share experiences with people in the same boat. Thank you all!

I could have never even started this PhD without the help of my former master thesis supervisor, Lydia Afman. I am very thankful that you helped me find a new PhD after my "adventure" in Spain!

Doing a PhD consumed a large part of my life and I would have never made it without the help and support from people outside of the institute and university.
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To my friends in the Netherlands, sorry for not being in touch as much as I wanted! I have missed all of you so much, and I cannot wait celebrate this with all of you. The PhD is almost over, I will be back in touch soon!

I don't think I even need to say this, but without the support of my family none of this would have been possible. Mama en papa, heel erg bedankt voor alles de afgelopen jaren. Ik weet dat het voor jullie niet altijd makkelijk was om een dochter in het buitenland te hebben wonen, maar met wat op en neer vliegen van beide kanten hebben we dat volgens mij redelijk goed opgelost. En het resultaat mag er zijn (al zeg ik het zelf), jullie dochter is straks Dr Blokker!

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This is it then, the end of my PhD thesis. I am ready for the next challenge, starting my job at DSM Nutritional Products. There is no doubt in my mind that I will carry everything I have learned during my PhD with me for the rest of my life, both professionally and personally.

Thank you all,
Britt

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Appendices

Thesis Britt Blokker

Appendix 1 Dietary composition

The diets low and high fat diets used in our murine study came from Research Diets. Below you can find the composition of these diets. For a full description of the vitamin and mineral mixes used see http://www.researchdiets.com/open-source-diets/stock-diets/dio-series-diets.

High fat D12451

Formula

Product #D12451	gm%	kcal%
Protein	24	20
Carbohydrate	41	35
Fat	24	45
Total		100
kcal/gm	4.73	

Ingredient	gm	kcal
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	72.8	291
Maltodextrin 10	100	400
Sucrose	172.8	691
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	177.5	1598
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Red Dye #40	0.05	0
Total	858.15	4057

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

*Typical analysis of cholesterol in lard = 0.72 mg/gram.
Cholesterol (mg)/4057 kcal = 127.8
Cholesterol (mg)/kg = 148.9

Low fat D12450H

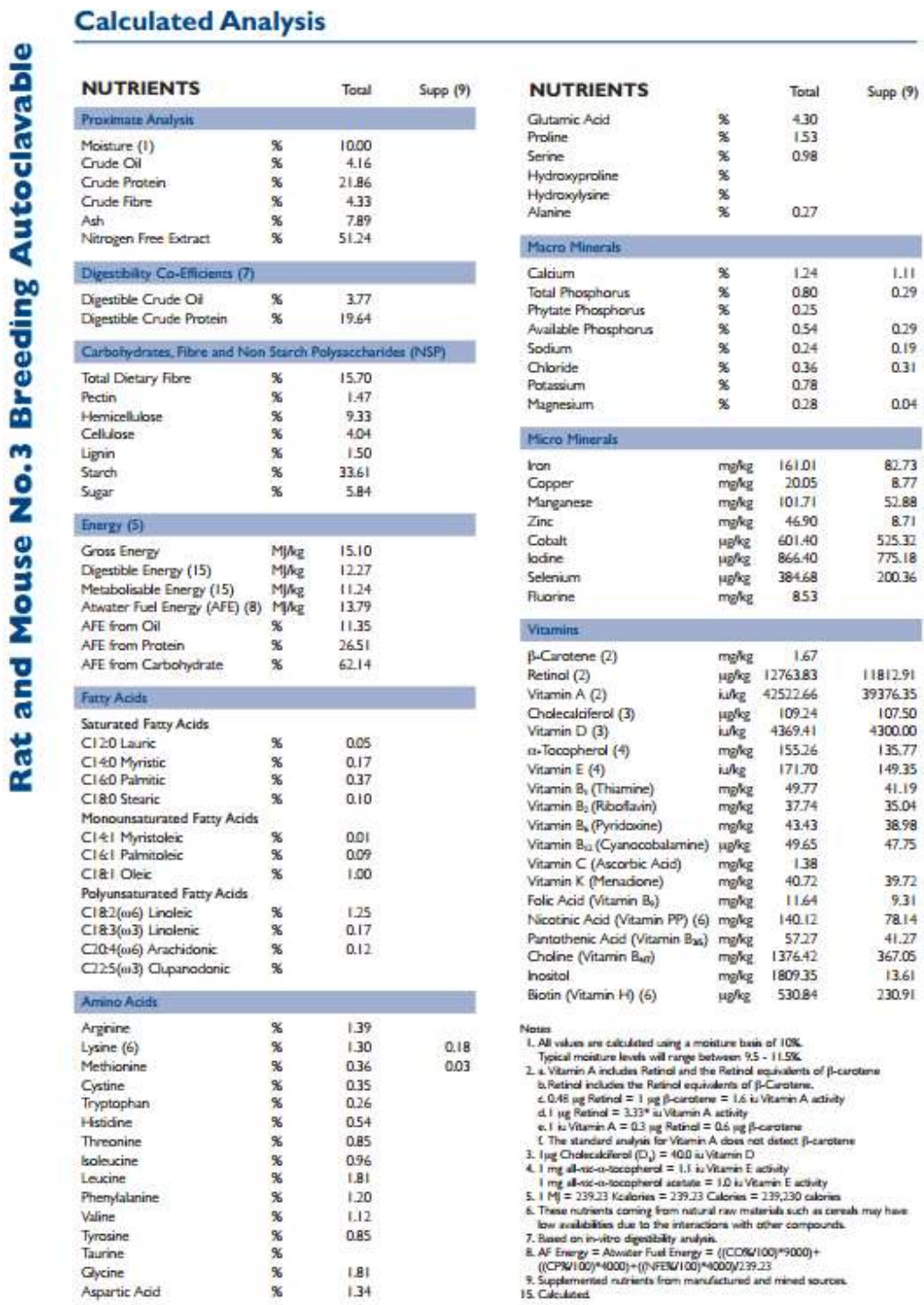
Formula

Product #D12450H	gm%	kcal%
Protein	19.2	20
Carbohydrate	67.3	70
Fat	4.3	10
Total		100
kcal/gm	3.85	

Ingredient	gm	kcal
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	452.2	1808.8
Maltodextrin 10	75	300
Sucrose	172.8	691.2
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	20	180
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Yellow Dye #5	0.04	0
FD&C Red Dye #40	0.01	0
Total	1055.05	4057

*Typical analysis of cholesterol in lard = 0.72 mg/gram.
Cholesterol (mg)/4057 kcal = 14.4
Cholesterol (mg)/kg = 13.6

In our murine studies a control group was put on the standard chow diet used in the Disease Modelling Unit of the University of East Anglia (SDS diet RM3, Dietex international, UK).



Rat and Mouse No.3 Breeding Autoclavable

Calculated Analysis

NUTRIENTS		Total	Supp (9)
Proximate Analysis			
Moisture (1)	%	10.00	
Crude Oil	%	4.16	
Crude Protein	%	21.86	
Crude Fibre	%	4.33	
Ash	%	7.89	
Nitrogen Free Extract	%	51.24	
Digestibility Co-Efficients (7)			
Digestible Crude Oil	%	3.77	
Digestible Crude Protein	%	19.64	
Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)			
Total Dietary Fibre	%	15.70	
Pectin	%	1.47	
Hemicellulose	%	9.33	
Cellulose	%	4.04	
Lignin	%	1.50	
Starch	%	33.61	
Sugar	%	5.84	
Energy (5)			
Gross Energy	MJ/kg	15.10	
Digestible Energy (15)	MJ/kg	12.27	
Metabolisable Energy (15)	MJ/kg	11.24	
Atwater Fuel Energy (AFE) (8)	MJ/kg	13.79	
AFE from Oil	%	11.35	
AFE from Protein	%	26.51	
AFE from Carbohydrate	%	62.14	
Fatty Acids			
Saturated Fatty Acids			
C12:0 Lauric	%	0.05	
C14:0 Myristic	%	0.17	
C16:0 Palmitic	%	0.37	
C18:0 Stearic	%	0.10	
Monounsaturated Fatty Acids			
C14:1 Myristoleic	%	0.01	
C16:1 Palmitoleic	%	0.09	
C18:1 Oleic	%	1.00	
Polyunsaturated Fatty Acids			
C18:2(ω6) Linoleic	%	1.25	
C18:3(ω3) Linolenic	%	0.17	
C20:4(ω6) Arachidonic	%	0.12	
C22:5(ω3) Clupanodonic	%		
Amino Acids			
Arginine	%	1.39	
Lysine (6)	%	1.30	0.18
Methionine	%	0.36	0.03
Cystine	%	0.35	
Tryptophan	%	0.26	
Histidine	%	0.54	
Threonine	%	0.85	
Isoleucine	%	0.96	
Leucine	%	1.81	
Phenylalanine	%	1.20	
Valine	%	1.12	
Tyrosine	%	0.85	
Taurine	%		
Glycine	%	1.81	
Aspartic Acid	%	1.34	

NUTRIENTS		Total	Supp (9)
Glutamic Acid	%	4.30	
Proline	%	1.53	
Serine	%	0.98	
Hydroxyproline	%		
Hydroxylysine	%		
Alanine	%	0.27	
Macro Minerals			
Calcium	%	1.24	1.11
Total Phosphorus	%	0.80	0.29
Phytate Phosphorus	%	0.25	
Available Phosphorus	%	0.54	0.29
Sodium	%	0.24	0.19
Chloride	%	0.36	0.31
Potassium	%	0.78	
Magnesium	%	0.28	0.04
Micro Minerals			
Iron	mg/kg	161.01	82.73
Copper	mg/kg	20.05	8.77
Manganese	mg/kg	101.71	52.88
Zinc	mg/kg	46.90	8.71
Cobalt	µg/kg	601.40	525.32
Iodine	µg/kg	866.40	775.18
Selenium	µg/kg	384.68	200.36
Fluorine	mg/kg	8.53	
Vitamins			
β-Carotene (2)	mg/kg	1.67	
Retinol (2)	µg/kg	12763.83	11812.91
Vitamin A (2)	iu/kg	42522.66	39376.35
Cholecalciferol (3)	µg/kg	109.24	107.50
Vitamin D (3)	iu/kg	4369.41	4300.00
α-Tocopherol (4)	mg/kg	155.26	135.77
Vitamin E (4)	iu/kg	171.70	149.35
Vitamin B_1 (Thiamine)	mg/kg	49.77	41.19
Vitamin B_2 (Riboflavin)	mg/kg	37.74	35.04
Vitamin B_6 (Pyridoxine)	mg/kg	43.43	38.98
Vitamin B_{12} (Cyanocobalamine)	µg/kg	49.65	47.75
Vitamin C (Ascorbic Acid)	mg/kg	1.38	
Vitamin K (Menadione)	mg/kg	40.72	39.72
Folic Acid (Vitamin B_9)	mg/kg	11.64	9.31
Nicotinic Acid (Vitamin PP) (6)	mg/kg	140.12	78.14
Pantothenic Acid (Vitamin B_{5R})	mg/kg	57.27	41.27
Choline (Vitamin B_{4R})	mg/kg	1376.42	367.05
Inositol	mg/kg	1809.35	13.61
Biotin (Vitamin H) (6)	µg/kg	530.84	230.91

Notes
1. All values are calculated using a moisture basis of 10%.
   Typical moisture levels will range between 9.5 - 11.5%.
2. a. Vitamin A includes Retinol and the Retinol equivalents of β-carotene
   b. Retinol includes the Retinol equivalents of β-Carotene.
   c. 0.48 µg Retinol = 1 µg β-carotene = 1.6 iu Vitamin A activity
   d. 1 µg Retinol = 3.33* iu Vitamin A activity
   e. 1 iu Vitamin A = 0.3 µg Retinol = 0.6 µg β-carotene
   f. The standard analysis for Vitamin A does not detect β-carotene
3. 1µg Cholecalciferol (D_3) = 40.0 iu Vitamin D
4. 1 mg all-rac-α-tocopherol = 1.1 iu Vitamin E activity
   1 mg all-rac-α-tocopherol acetate = 1.0 iu Vitamin E activity
5. 1 MJ = 239.23 Kcalories = 239.23 Calories = 239,230 calories
6. These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
7. Based on in-vitro digestibility analysis.
8. AF Energy = Atwater Fuel Energy = ((CO%/100)*9000)+((CP%/100)*4000)+((NFE%/100)*4000)/239.23
9. Supplemented nutrients from manufactured and mined sources.
10. Calculated.

Appendix 2 Bile acid standards, LC-MS conditions and mass fragmentation monitoring values for bile acid measurements

Bile acid standards: d4 Internal standards

Each d4 internal standard is prepared at 1 mg/ml in MeOH

Prepare 5 solutions of these all at 40 µg/ml by taking 400µl of each stock standard to 10 ml in 70% MeOH

INT Std #	Int Std(s)
1	d4-GCA + d4 LCA
2	d4-CA
3	d4-CDCA
4	d4-DCA
5	d4-DCA/CDCA/CA/GCA/LCA

Calibration standards

Each bile acid is prepared at 1 mg/ml in MeOH and stored refrigerated.

A 10 µg mix of the BAs is prepared in 70% MeOH by taking 100 µl of each individual bile acid (at 1 mg/ml) into a pooled vial and making to 10 ml total volume. Make all to total volume of 500µl with methanol.

Std (ng/ml)	Vol (µl)	Of what std	Make up vol (µl) (MeOH)	Notes
4000	200	10 µg/ml mix	300	
2000	100	10 µg/ml mix	400	
1000	50	10 µg/ml mix	450	Prep x 2
500	25	10 µg/ml mix	475	
200	10	10 µg/ml mix	490	
100	50	1000 ng/ml mix	450	Prep x 2
25	12.5	1000 ng/ml mix	487.5	

15	75	100 ng/ml mix	425	
10	50	100 ng/ml mix	450	
5	25	100 ng/ml mix	475	
0	0	-	500	

Then to each of these, add 25µl of d4-Int Std mix #5 (40 µg/ml) to give 2000 ng/ml each.

LC-MS

Conditions

Column: Supelco Ascentis Express C18 150 x 4.6, 2.7µm

Flow: 600 µl/min

Mobile phase A: Water + 5mM Amm. Ac + 0.012% Formic acid

Mobile Phase B: Methanol + 5mM Amm. Ac + 0.012% Formic acid

Inj: 5µl

Column: 40°C

Mobile phase preparation

In one litre of MeOH or Water:

5 mM Ammonium Acetate = 0.385g

0.012% formic acid = 120 µl

LC Gradient

Time	%B
0	50
2	50
20	95
24	95
25	50
29	50

Source Conditions (negative mode)

SOURCE	
CUR:	25
TEM:	550
GS1:	40
GS2:	50
ihe:	ON
CAD:	-2

IS:	-4500
EP	-10
CXP	-9

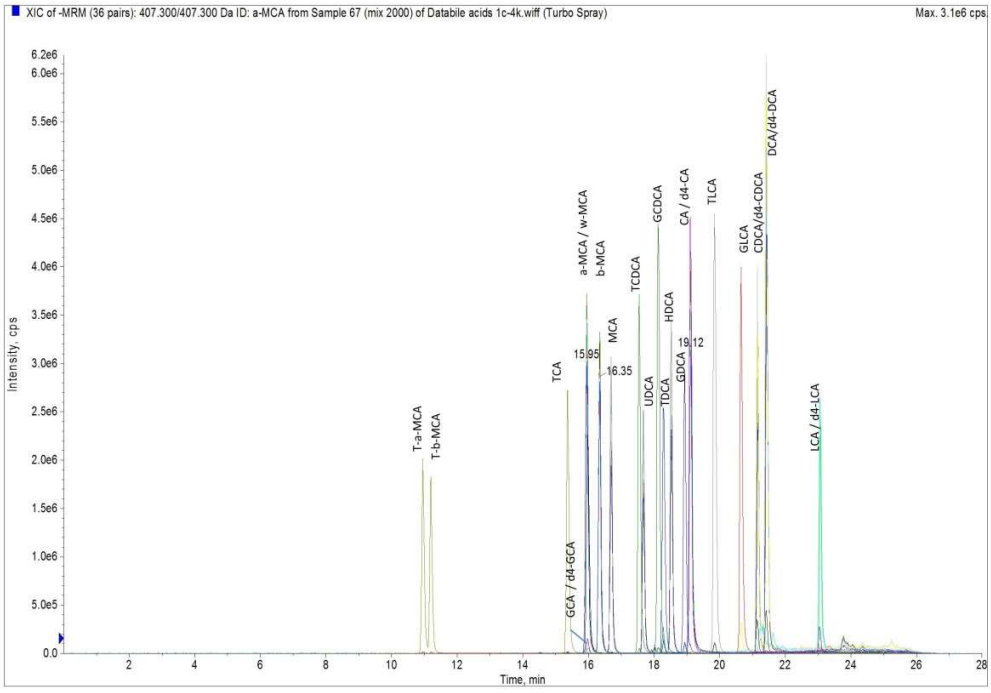
MRM settings

ID	Q1	Q3	Dwell	DP	CE	RT
LCA	375.3	375.3	20	-90	-10	22.70
CDCA	391.3	391.3	20	-120	-10	20.85
DCA	391.3	391.31	20	-120	-10	21.17
HDCA	391.3	391.32	20	-120	-10	18.29
MCA	391.3	391.33	20	-120	-10	16.57
UDCA	391.3	391.34	20	-120	-10	17.52
a-MCA	407.3	407.3	20	-120	-10	15.93
b-MCA	407.3	407.31	20	-120	-10	16.29
CA	407.3	407.32	20	-120	-10	18.89
GLCA	432.3	432.3	20	-80	-10	19.77
GCDCA	448.3	448.31	20	-80	-10	17.72
GDCA	448.3	448.32	20	-80	-10	18.28
GCA	464.3	464.3	20	-80	-10	15.70
TLCA	482.2	482.2	20	-130	-10	19.19
TCDCA	498.3	498.3	20	-130	-10	17.10
TDCA	498.3	498.31	20	-130	-10	17.64
T-a-MCA	514.3	514.3	20	-130	-10	10.81
T-b-MCA	514.3	514.31	20	-130	-10	11.06
TCA	514.3	514.32	20	-130	-10	15.08
d4-LCA	379.3	379.3	20	-90	-10	22.70
d4-CDCA	395.3	395.31	20	-120	-10	20.83
d4-DCA	395.3	395.3	20	-120	-10	21.13
d4-CA	411.3	411.3	20	-120	-10	18.86
d4-GCA	468.4	74	20	-80	-40	15.71
P lipid query	153	153	20	-130	-10	

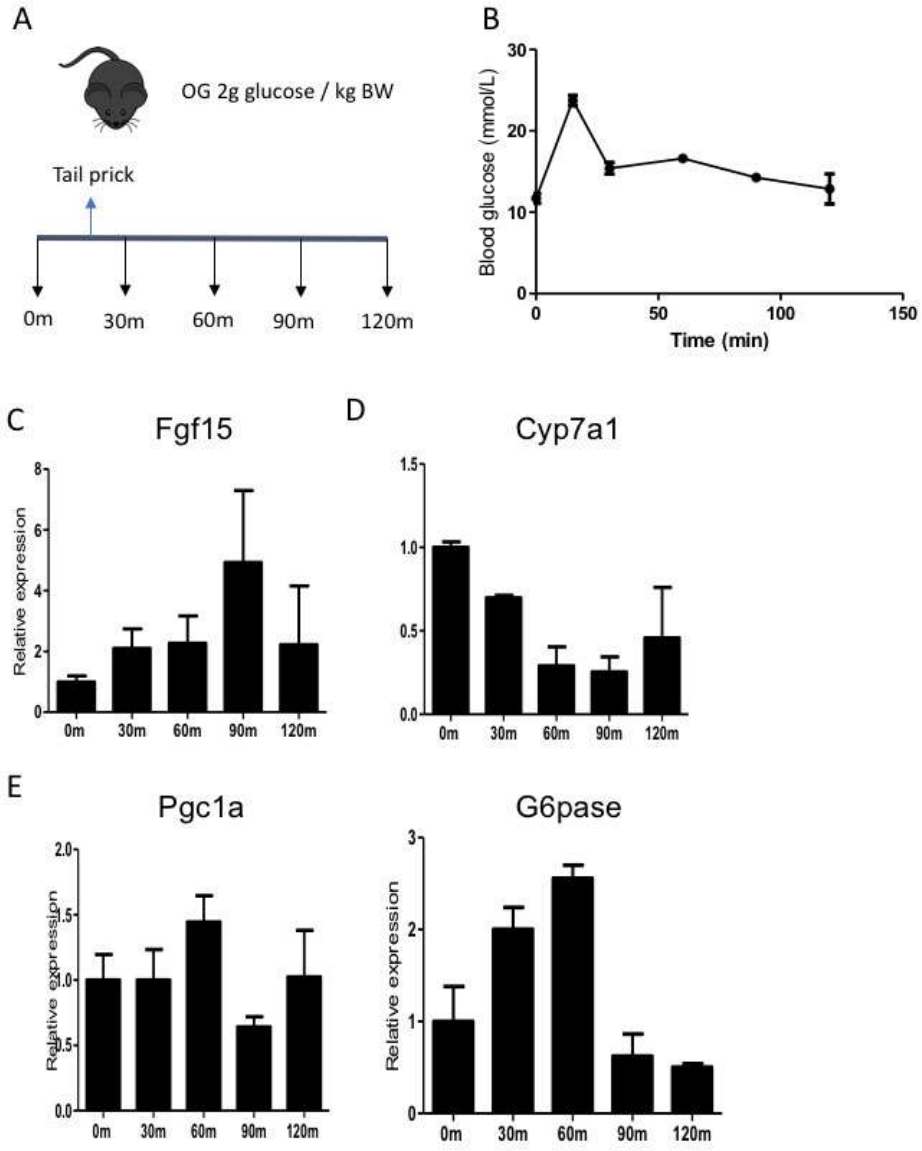
Qualifiers

ID	Q1	Q3	Dwell	DP	CE	RT
GLCA	432.3	74	20	-80	-40	20.59
GCDCA	448.3	74.11	20	-80	-40	18.31
GDCA	448.3	74.12	20	-80	-40	18.93
GCA	464.3	74	20	-80	-40	16.03
TCDCA	498.3	80.1	20	-130	-60	17.33
TDCA	498.3	80.11	20	-130	-60	17.87
T-a-MCA	514.3	80.1	20	-130	-60	10.68
T-b-MCA	514.3	80.11	20	-130	-60	11.02
TCA	514.3	80.12	20	-130	-60	15.12
TLCA	482.2	80	20	-130	-60	19.57

QTrap method file: **Bile salts (MP).dam**



Appendix 3. Oral gavage of glucose leads to a time dependent increase of Fgf15 expression in the ileum and corresponding hepatic glucose regulation.



Appendix 3 Oral gavage of glucose leads to a time dependent increase of Fgf15 expression in the ileum and corresponding hepatic glucose regulation. Mice received a glucose load of 2g / kg BW via oral gavage (OG) and two mice per time-point were sacrificed at 0, 30, 60, 90 and 120 minutes after OG, blood glucose was measured at all time points and additionally at 15 minutes via a tail prick (A). Blood glucose shows an initial increase and consequent normalization after glucose OG (B). Ileal Fgf15 expression increases 90 minutes after the glucose OG (C), while hepatic Cyp7a1 decreases (D). Hepatic glucose metabolism genes known to respond to FGF15/19 decrease in expression after 90 minutes, correlating with the increase in Fgf15 expression (E). *Due to the low number of mice no statistical analysis could be performed.*