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Enrichment of gastrointestinal anaerobes by a novel technique

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This thesis was commissioned by a Finnish company Alimetrics Ltd., which specializes in life sciences. The aim was to isolate bacterial communities of broiler chicken gastrointestinal tract while segregating them by their preferred source of carbon. Successful execution of this novel approach would allow for significant further research potential.

During this thesis a novel method for isolating anaerobic bacteria was developed. The principal idea was to microbiologically enrich gastrointestinal bacteria by culturing them on specific growth medium.

Anaerobic cultivation on selective medium was performed to enrich seven different cultures. Inoculum was gastrointestinal bacteria of broiler chicken. Bacteria was cultured in liquid growth media. Growth media was otherwise identical except the difference of included carbon source. Seven different carbon sources were used to enrich bacteria that could utilize them. Substrates used were various carbohydrates, protein, fat and lactic acid. Bacteria was propagated every day, which diluted the bacterial population that could not utilize the particular substrate. Enrichment cultures were subject to short-chain fatty acid composition, ammonium concentration, and bacterial composition analysis.

The novel method proved to be adequate and multiple cultures with specific characteristics were isolated. To a surprise, the genus *Bacteroides* was abundant in most cultures, which could be explained by the composition of used substrates. Metabolite analyses indicated features that are widely associated with bacterial utilization of each carbon source in question.

The enrichment part of experiment was successful and potential for further research is significant. However, a method for treating substrates to simulate digestion of small intestine before enrichment would be advantageous.

Keywords	anaerobes, gastrointestinal bacteria, isolation, enrichment, short-chain fatty acid, metabolites, digestion, absorption, qPCR

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Tämä insinöörityö on tehty Alimetricsin toimeksiannosta. Alimetrics on biotieteisiin keskittyvä tutkimusta tekevä suomalainen yritys. Päämääränä oli broilerin suolistomikrobiryhmien rikastaminen sekä erottelu hiilenlähdepreferessien mukaisesti. Onnistuminen mahdollistaisi merkittävät jatkotutkimusmahdollisuudet.

Insinöörityön aikana kehitettiin uusi menetelmä, jolla pystytään eristämään anaerobisia bakteereita. Keskeinen ajatus on mikrobiologinen rikastus, joka on yksinkertaistettuna bakteerien kasvatus ja propagointi spesifeillä kasvualustoilla.

Anaerobisia suolistobakteereita viljeltiin seitsemällä spesifillä nestemäisellä kasvualustalla. Bakteerit olivat peräisin broiler-kanan umpisuolesta. Kasvatusalustat olivat identtisiä, lukuun ottamatta lisättyä hiilenlähdettä. Hiilenlähteinä käytettiin erilaisia hiilihydraatteja, proteiinia, rasvaa sekä maitohappoa. Bakteeripopulaatiot siirrostettiin uudelleen vuorokauden välein, mikä laimensi kasvualustan hiilenlähdettä hyödyntämättömät bakteerit. Rikasteet karakterisoitiin analysoimalla rasvahappokoostumus, ammoniumkonsentraatio sekä bakteerikoostumus.

Kehitetty rikastusmenetelmä osoittautui toimivaksi, ja näin ollen seitsemän eri ominaisuuksia ilmentävää bakteeriyhdyskuntaa saatiin eristettyä. *Bacteroides*-suku oli kaikista runsaslukuisin useimmassa viljelmässä, minkä voi selittää rikastamiseen käytettyjen substraattien ominaisuuksilla. Metaboliittianalyysit vahvistivat kokeen onnistumisen, sillä bakteerit fermentoivat erityyppisistä substraateista odotetun kaltaisia tuotteita.

Työn rikastusosio oli onnistunut, ja jatkotutkimuksien mahdollisuudet ovat merkittävät. Substraatteja varten tulisi kuitenkin kehittää esikäsittelymenetelmä, jolla pystyisi simuloimaan ohutsuolen suorittaman kaltaista ruuansulatuskäsittelyä.

Avainsanat	anaerobiset bakteerit, suolistobakteerit, eristys, rikastus, rasvahapot, metaboliitit, ruuansulatus, qPCR

Contents

Abbreviations

ATP	Adenosine triphosphate
Bp	Basepairs
BCFA	Branched-chain fatty acid
cfu	Colony forming units
cDNA	Complimentary DNA
dsDNA	Double stranded deoxyribonucleic acid
HCl	Hydrochloric acid
IMB	Intestinal mucosal barrier
mM	Millimolarity
mV	Millivolts
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
RS	Resistant starch
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RT	Reverse transcription
SCFA	Short-chain fatty acid
SOD	Superoxide dismutase
VFA	Volatile fatty acid

1 Introduction

This thesis was a study commissioned by Alimetrics Ltd. The main goal was to isolate and segregate bacterial communities by their preference of carbon source. Enrichment cultures were subsequently subjected to characterization. A novel method for enriching fastidious anaerobic microbes of gastrointestinal tract was developed and multiple analyses were performed.

Molecular biology, specifically quantitative real-time polymerase chain reaction (qPCR) has become an essential laboratory technique for studying the gastrointestinal microbiota of human and animals. Microbial 16S rRNA genes are regularly subject to studies of evolutional and genomic diversity of microorganisms. The typical approach is to extract fecal DNA of control and study specimens. Genes of particular interest are then amplified using qPCR technique and resulting quantities are compared for conclusions. qPCR is rapid, sensitive and robust method. Additionally, mRNA (messenger RNA) can be extracted for gene expression studies. Reverse transcription is applied to purified mRNA to obtain cDNA (complimentary DNA). Subsequently, qPCR is used for amplification of cDNA. For example, it is possible to examine acceleration of antimicrobial peptide expressions during an infection (Mohammed *et al*. 2016). qPCR and RT-qPCR (reverse transcription qPCR) are relatively fast methods and can yield precise information of strain specific content and cellular activity. However, a major drawback of using qPCR techniques is the requirement of prior knowledge regarding the microbial nucleic acid sequences present in the sample. To sequence the full genome of a microorganism, it is required to successfully isolate its DNA at first. Even though the next generation sequencing has made it fast and affordable to determine precise order of nucleotides within the chromosomal DNA of microorganism, there are several microbes that have never been successfully isolated as a pure culture. Whole genome shotgun sequencing analysis of the gut microbiota composition could yield helpful results, but its size exceeds the size of a human genome (Sunneburg *et al*. 2004). Scientists may often find themselves staring at a vast sequence that is taxonomically far from being familiar. To overcome the challenge, a different approach is suggested. That is to focus not on a pure culture of single species but on a community of microorganisms that share particular characteristics and live communally. This fraction of gut microbiota can be then sequenced, characterized and used for further studies.

To achieve the aim of this thesis, it is required to investigate and successfully execute methods of traditional microbiology. Laborious and challenging techniques of cultivating strictly anaerobic bacteria are nowadays often excluded from curriculums of learning institutions. For someone looking into anaerobic methods, the advice is to have a demonstration at a laboratory where anaerobic cultivation is routinely in use, as some steps of handling anaerobes are frequently difficult to master. Not only manual labor is demanding, but designing and compiling an environment that will keep microbial population thriving is tedious. Anaerobic microbes are typically fastidious to a greater extent when compared to aerobes, hence anaerobes require rich media for cultivation. To isolate gastrointestinal microorganisms, it is important to consider various metabolites of microbial community members. Microbes in nature appear as groups that benefit from each other (metabolic cross-feeding). Thus, when separated they are unable to survive, rendering traditional pure-culture methods ineffective. As most of bacteria fail to grow under artificial environments, sequencing the DNA of pure cultures is inconclusive approach; therefore, we need to study metabolic functions of the gut microbiota as communal actions carried out by groups, not a single individual microorganism. (Apajalahti *et al*. 2004)

Enrichment-isolation method that is based on feeding various carbon sources to microbes, has been applied to study numerically abundant but difficult-to-culture environmental aerobes (Wawrik *et al*. 2005). The principle of feeding not a complex but specific substrate, to enrich a community of microorganisms that can utilize chosen carbon source should work with anaerobes as well. Coupling enrichment-isolation with modern molecular and biochemical analyses will allow us to cover the chosen phenomenon comprehensively. For example, it is possible to combine enrichment of the gastrointestinal microbiota with next-generation sequencing and short-chain fatty acid analysis. As a whole, the experiment would yield DNA sequences that can be associated with the digestion of chosen nutrients and short-chain fatty acid composition introduced to GI tract by said metabolic activity. The enrichment-isolation technique, if executed successfully, enables exclusive insight that will be complemented by 16S rRNA gene based studies.

2 Physiology of gastrointestinal tract

The gastrointestinal (GI) tract i.e. alimentary canal is a muscular tube system, which in result of food digestion produces nutrients and energy while expelling remaining waste as feces and urine. Disintegration of food and absorption of nutrients occurs in several

phases throughout the canal but mainly in the small intestine. Mechanical and chemical breakdown are both applied in order to process food efficiently. Function of the GI tract is approximately similar across vertebrates. Major organs that participate in food digestion like pancreas and liver as well as the main parts of the GI tract such as duodenum, jejunum, ileum and cecum are found in both humans and chickens [Figure 1]. However, notable differences are for instance in mechanical breakdown of food: humans will chew food upon ingestion, whereas for chickens, gizzard, the site of mechanical grinding is located after crop. Crop functions as a food storage that passes it out for digestion. Breakdown of nutrients is catalyzed mainly by pancreatic hydrolases in small intestine. Nutrients absorb through intestinal cells that are called enterocytes and the main absorptive site is jejunum. Enterocytes have apical surface and are covered with microvilli, which houses digestive enzymes that assist in nutrient breakdown as well as membrane transport proteins which regulate the nutrient uptake.

Figure 1 Overview of parts and main organs of chicken (left) and human (right) gastrointestinal tract. Due to explanatory reasons, the body parts are slightly out of proportion. (Ülle 2017)

It is important to understand the food digestion that is result of host's metabolism in contrast to the function of gut microbiota that benefits the host. Albeit most of food digestion takes place in small intestine, the large intestine has the largest microbial content: 10^{11} organisms per gram wet weight respectively (Apajalahti & Kettunen 2006). The digestion of cellulose and other plant material that is performed by microorganisms and occurs in cecum and large intestine is called hindgut fermentation. It is suggested that the relationship between gut microbiota and the host is symbiotic in several ways. The relationship between the host and the gut microbiota can be commensal, which means that the product of microbe's metabolism can be used by the host for a beneficial effect. Mutualism is

obligatory and both, the host and the microbe have to benefit from this type of interaction. Microorganisms of GI tract ferment unused energy substrates to train host's immune system via SCFA end-products such as propionate, acetate and butyrate. Propionate upregulates T cells, increasing their ability to recognize foreign materials. Acetate reduces inflammations associated to colitis, arthritis, and even asthma (Willey *et al*. 2013). Butyrate is absorbed by the colonic mucosal cells, for which it provides a significant energy source. (Wong *et al*. 2006) However, products of gut microbiota are not always beneficial and compounds that are harmful at high concentrations might be produced from resistant protein (Apajalahti & Vienola 2016).

The GI tract features histological organization that is similar across the whole tract and is lined by a mucous membrane. Lumen is the interior space of GI tract, which encloses partly digested food i.e. digesta. In addition to commensal microbes, digesta comprises pro-inflammatory microorganisms, toxins and antigens. Intestinal mucosal barrier (IMB) prevents the uncontrolled translocation of harmful luminal contents from intestine while maintaining the ability to absorb nutrients. Intestinal mucosal barrier is significant for health and its dysfunction has been associated with food allergies, celiac disease, diabetes and inflammatory bowel disease (Groschwitz, Hogan 2009). The IMB consists of mucus gel layers that are formed of mucin molecules. Mucin molecules are protein glycoconjugates secreted by goblet cells. Mucins house oligosaccharide side chains that act as a decoy. The bacteria will be immobilized by binding to oligosaccharide chains using adhesins. This will prevent the bacteria from reaching and damaging epithelium. (Cornick *et al*. 2015)

2.1 Digestion and absorption of nutrients

2.1.1 Protein

Most proteolytic enzymes of GI tract are secreted as inactive forms (zymogens); they act as precursors before transforming to fully active form. This mechanism is to ensure that cells wherein the enzyme is produced are not digested themselves. Breakdown of protein into large peptides in stomach is catalyzed by pepsin. Pepsinogen, which is zymogen of pepsin, is released from gastric chief cells of stomach wall. Pepsinogen's structure unfolds in presence of HCl (hydrochloric acid), which exposes short amino acid chain for cleavage. Pepsinogens autocatalyze the removal of these amino acid chains, which

transforms them to pepsins, the fully active form [Figure 2]. Parietal cells located in the gastric glands secrete the necessary HCl. (Vander *et al*. 2001) Presence of HCl in stomach contributes to autocatalytic activation of pepsin and offers suitable pH conditions for enzymatic breakdown of dietary protein to large polypeptides in stomach.

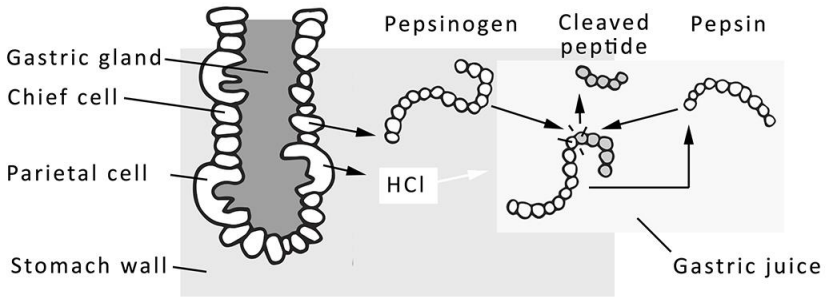

Figure 2 Production and activation of pepsin. HCl catalyzes the autoactivation of pepsin. Pepsinogen is produced by chief cells while HCl is secreted from parietal cells. In hydrochloric environment pepsinogen will reveal an amino chain. This amino chain is cleaved off by pepsin. (Ülle 2017)

Large polypeptides advance to small intestine's duodenum, which is the main site for protein digestion and absorption. Pancreas secretes pancreatic juice into the lumen of duodenum. Secretion contains trypsinogen, chymotrypsinogen and precarboxypeptidase zymogens. To transform trypsinogen into fully active trypsin, a membrane-bound brush border enzyme enterokinase that is located in microvilli, cleaves short amino acid chain from the end of trypsinogen. Trypsin has vital role as it transforms chymotrypsinogen and precarboxypeptidase to their active forms. To activate chymotrypsin, trypsin cleaves chymotrypsinogen transforming it to active π-chymotrypsin which carries on and auto-activates π-chymotrypsin to δ-chymotrypsin which subsequently auto-activates itself into final form the α-chymotrypsin. [Figure 3] Unlike trypsin and chymotrypsin, the carboxypeptidase hydrolyses peptide bonds specifically at carboxyl end of protein. Digestion of long polypeptides by pancreatic proteinases in duodenum results in short polypeptides as well as di- and tripeptides, which the host needs to break further in order to absorb them.

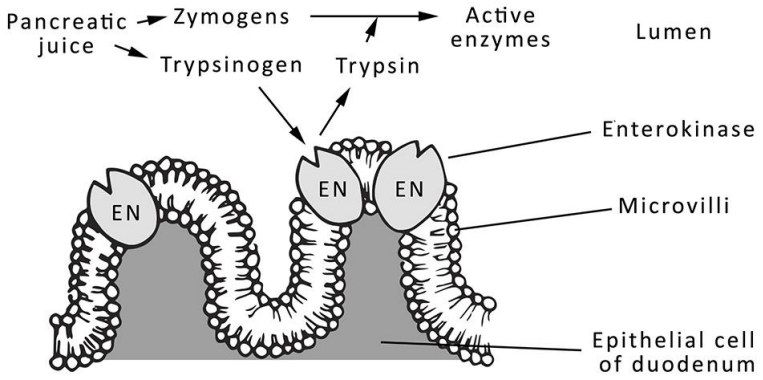

Figure 3 Activation scheme for pancreatic proteases. Brush border enzyme enterokinase activates trypsin, which subsequently activates chymotrypsinogen and precarboxypeptidase. (Ülle 2017)

Last part of host's protein digestion occurs on microvilli-covered surface of intestinal epithelial cells i.e. brush border membrane. In microvilli are located aminopeptidase, dipeptidase and carboxypeptidase enzymes. Aminopeptidase hydrolyses peptide bond from amino terminal of the protein while di- and carboxypeptidases cleave accordingly. Di- and tripeptides near the membrane surface can be absorbed to enterocyte by co-transport with proton (H^+). Absorbed peptides are then digested into amino acids by cytoplasmic peptidases and diffused into the bloodstream. Peptides that are digested to amino acids already in the lumen are absorbed to enterocyte by co-transport with sodium and subsequently are diffused into the bloodstream.

Several types of protein can bypass small intestine and escape host's digestive system. The resistant protein of ingested origin, for example the soluble protein as well as the small insoluble particles of protein can reach the cecum. In addition, microbial protein and host-synthesized endogenous protein (enzymes, epithelial cells, antibodies etc.) can penetrate the interdigitating meshwork of villi and musculature that acts as a filtrating gate to cecum. In the cecum, the protein-fermenting putrefactive bacteria can produce potentially harmful end-products from aromatic amino acids. At increased concentrations, the end-product of tryptophan fermentation (skatole) is known to inhibit production of adenosine triphosphate (ATP). The amino acid tyrosine is fermented to *p*-cresol and phenol and will result in similar harmful function as fermentation of tryptophan. Other end-products of protein fermentation in cecum include straight-chain volatile fatty acids as well as branched chain fatty acids (BCFA) which are not known to be toxic (Apajalahti & Vienola 2016).

2.1.2 Carbohydrates

Typical ingested carbohydrates vary by their molecular size and consist of disaccharides of which table sugar is the usual example, oligosaccharides such as some of dietary fibers, and polysaccharides (starch and dietary fibers). All of the carbohydrates need to be hydrolyzed to monosaccharides before absorption. Upon ingestion and mechanical breakdown of food (chewing), α-amylase enzyme in saliva of human oral cavity will start to hydrolyze starch into smaller low-molecular-weight polysaccharides called α-dextrins. This is continued down until stomach where the amylase is denatured by gastric acid (Smith *et al*. 2003). Chicken are different; and upon ingestion, the feed will transport intact down the esophagus to the crop. Esophagus is lubricated by secreted mucus, which assists in transportation of feed. There is no considerable absorption of nutrients or secretion of enzymes in crop. However, its contents are gradually moistened which aids the grinding and enzymatic digestion further down in the digestive tract. (Svihus 2014) Breakdown of polysaccharides in human small intestine is performed by pancreatic α-amylase. The α-amylase produces di- and trisaccharides as well as limit dextrins which cannot be absorbed. Limit dextrins are non-reducing dextrins and require additional enzyme for further hydrolysis. Resulting products of α-amylatic hydrolysis are broken further down to monosaccharides by brush-border enzymes located in microvilli [Figure 4]. The resulting glucose enters intestinal cells via co-transport with sodium, by the process of secondary active transport.

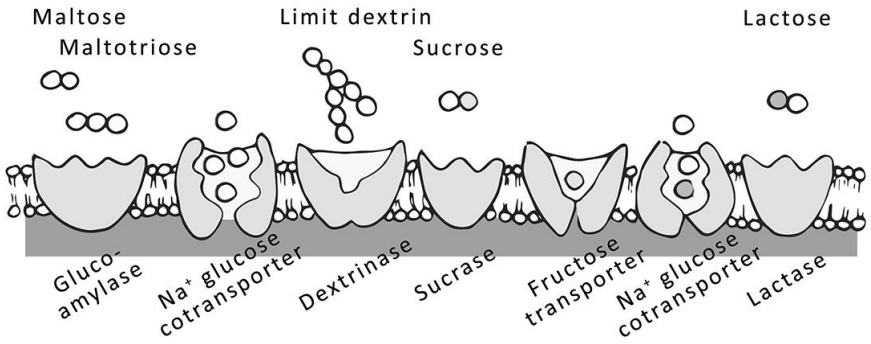

Figure 4 Digestion and absorption of carbohydrates after hydrolysis by salivary and pancreatic amylases. Maltose and maltotriose are hydrolyzed by glucoamylase. Dextrins are broken down by dextrinase. Sucrose and lactose are broken down by sucrose and lactase respectively. All monosaccharides except fructose are absorbed via Na^+ glucose co-transporters. Fructose has an exclusive transporter. (Ülle 2017)

The process is named active transport because the monosaccharides are transported against their concentration gradient which requires energy. The energy for secondary

active transport is gained from another molecule that is moving down its concentration gradient: the molecule will bond with a monosaccharide and will transport it against its concentration gradient inside the cell. Fructose uses different method of transport, which is not completely understood. However, it is thought to be transported inside cells via facilitated diffusion, which is use of channel protein as a gateway to move down the concentration gradient. Thus, the fructose is thought to be absorbed via passive transport (Smith *et al*. 2003).

Not all of the ingested carbohydrates will digest in small intestine. Some starches like corn starch with high amylose content, or very dry starch (e.g. starch in dried beans), as well as dietary fiber, and to a lesser extent undigested sugars can enter the colon. The importance of carbohydrates that pass small intestine is accounted for health-promoting fermentation products of saccharolytic colonic bacteria. Beneficial products are SCFAs and lactate. The principal SCFAs are acetate, propionate and butyrate. Acetate is transported to liver and can act as a substrate for cholesterol synthesis. Butyrate is absorbed by the colonic mucosal cells, for which it provides a significant energy source. Propionate metabolism is less well understood and much of the knowledge comes from studies in ruminants (Wong *et al*. 2006). Dietary fiber, which is typically a shorter carbohydrate chain when compared to starch, consists of plant material that is polysaccharide derivative and lignin. Dietary fibers can be categorized by their solubility in water. Examples of soluble fiber are pectic substances (e.g. arabinoxylans, β-glucans), gums (e.g. arabinogalactans, galactomannans) and mucilages (branched and substituted galactans). Certain types of soluble fiber are associated with disease prevention. Pectins for example may lower blood cholesterol levels by binding bile acids. However, fiber-associated beneficial effects are relatively specific for the type of fiber and the physical form of the fiber origin (Smith *et al*. 2003). Starch that escapes the small intestine is referred to as resistant starch (RS). Resistant starches are subdivided into four categories: RS_1. RS_2, RS_3 and RS_4. These categories define the level of resistance to digestion in small intestine. The RS_1 represents starch that is not readily available to digestive enzymes because it is physically protected inside of partly milled grains of whole grains. RS_2 has compact structure where starch is tightly packed in radial pattern and is relatively dehydrated (e.g. ungelatized starch). Thus, RS_2 is digested very slowly and incompletely in small intestine. The RS_3 represents the most resistant naturally occurring starch and is entirely resistant to pancreatic amylases (e.g. retrograded starch). RS_4 includes starches that are chemically modified to resist digestion (e.g. distarch phosphate ester) (Sajilata *et al*. 2006).

2.1.3 Lipids

The digestion of dietary fat can be addressed as the breakdown of triglycerides to fatty acids and monoglycerides. Triglycerides have weak solubility in water and are esters of three fatty acids and glycerol. Upon ingestion of fat-containing nutriment, the limited breakdown of lipids in the human GI tract starts immediately in the mouth by lingual lipase and continues down in stomach by gastric lipase. Lingual lipase is secreted in oral cavity along with saliva (Smith *et al*. 2003). There have been no reports of lipases present in upper GI tract of poultry (Krogdahl 1985). When dietary fat enters its major digestion site, the duodenum of small intestine, it is emulsified by bile salts. It is to be noted that breakdown of fat in mouth by lingual lipase does not need to be catalyzed by bile salts. Bile salts are synthesized in the liver and contain hydrophobic and hydrophilic compounds. They are secreted into intestinal lumen via gallbladder. Bile salts catalyze the hydrolysis of lipids by clinging to triglyceride molecules, which causes the breakdown of fat globules to smaller droplets. As a result, it will increase the surface area of globule contents which will increase the solubility of ingested fat. The principle lipid digesting enzyme is pancreatic lipase, which breaks down triglycerides to one monoglyceride and two free fatty acids. Pancreatic lipase is assisted by colipase which is activated by trypsin. Colipase prevents inhibitory effect of bile salts. After hydrolysis, fatty acids and monoglyceride are ferried by bile salt-micelles for absorption through microvilli. Bile salts are not absorbed and are left behind in the lumen and recirculated through ileum by a process called enterohepatic circulation. Fat-soluble vitamins and other dietary lipids such as cholesterol and lysophospolipids are packed into ferrying micelles as well. Short- and medium-chain fatty acids (C4 to C12) do not need to be packaged into bile salt because their solubility does not need to be increased (Smith *et al*. 2003). Uptake of lipids into epithelial cells has been suggested to occur not only through protein channels, but independently as well, without help of proteins. This means that share of lipid absorption does not need special transport means like facilitative diffusion or active transport. Once inside epithelial cell, monoglyceride is further hydrolyzed to glycerol and free fatty acid. Products of complete triglyceride hydrolysis, with the help of fatty acid-binging proteins, must traverse the cytoplasm to reach the endoplasmic reticulum, where they are used as building blocks for synthesis of complex lipoproteins i.e. chylomicrons. Chylomicrons will transport the dietary lipids around the body (Iqbal & Hussain 2009).

3 Anaerobes

3.1 Cellular respiration

The key distinction between aerobes and anaerobes lies in the difference of energy production i.e. generation of ATP (adenosine triphosphate). In aerobic respiration, glycolysis is the first stage. Glycolysis is a pathway that occurs in cytosol and converts six-carbon glucose to pyruvate. This happens in ten steps catalyzed by ten different enzymes and will result in two three-carbon pyruvates. Glycolysis is followed by decarboxylation of pyruvate where acetyl-coenzyme A is formed in result of combining acetyl group (decarboxylated pyruvate) with coenzyme A. The third step is Krebs cycle or more commonly citric acid cycle which takes place in mitochondria. The principle of Krebs cycle is in continuous consumption and generation of citric acid through various enzyme-catalyzed steps. Finally, most of ATP is generated from electron transport chain (ETC). In ETC, membrane-bound protein complexes pass electrons gained from NADH, which was previously generated in Krebs cycle. As electrons pass the protein complexes of transport chain, they pump protons from mitochondrial matrix into the membrane space. At the end of ETC of aerobic respiration, the final electron acceptor is oxygen. The ATP is generated when the protons will float back from membrane space into the mitochondrial matrix by electrochemical gradient through ATP synthase (Willey *et al*. 2013).

In the anaerobic respiration, terminal electron acceptors of ETC are molecules such as sulfate (SO_4^{2-}) and nitrate (NO_3^-). These molecules have a lower reduction potential in contrast to aerobic respiration (Willey *et al*. 2013). Most anaerobes of large intestine are obligate. This means that they are unable to utilize oxygen for growth and will die upon exposure to even low levels of oxygen. Molecular oxygen (O_2) contains two unpaired electrons in its outer orbital. Therefore, it is readily reduced to superoxide (O_2^-). Death of the anaerobic cell can be caused by superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl (OH) which are all free radicals. These three radicals are formed from partial reduction of molecular oxygen by gaining electrons [Equation 1].

$$O_2 \xrightarrow{+1e^-} O_2^- \xrightarrow{+1e^-} H_2O_2 \xrightarrow{+1e^-} OH \xrightarrow{+1e^-} H_2O \quad (1)$$

Free radicals are highly reactive and for this reason can interfere and disrupt many cellular processes i.e. cause oxidative stress (McCord 2000). Atherosclerosis, inflammatory

conditions and even certain cancers can be sourced to oxidative stress. Aerobic and facultative anaerobic organisms have enzymes like superoxide dismutase (SOD) which deal with this problem. The SOD catalyzes dismutation of the superoxide into hydrogen peroxide (H_2O_2) [Equation 2] and catalase further decomposes hydrogen peroxide into water (H_2O) and oxygen (O_2) [Equation 3] (Young & Woodside 2001).

$$2O_2^- + 2H^+ \xrightarrow[dismutase]{superoxidase} H_2O_2 + O_2 \quad (2)$$

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad (3)$$

The described protective cellular mechanism is lacking or insufficient in anaerobic organisms (Hentges 1996). Thus, it is crucial to retain anoxic (anaerobic) conditions during handling of obligate anaerobic microorganisms.

3.2 Fermentation

In an oxygen-free environment obligate or facultative anaerobic bacteria are able to carry fermentation to gain energy. There are several fermentation pathways producing various metabolites, yet their primary function, similarly to respiration is the generation of ATP. Respiration and fermentation both include glycolysis. Major difference is that the electron transport chain is absent in fermentation, thus, less ATP is produced. Typically, fermentation produces acids or alcohol and different pathways are named after the major non-ATP product. Intestinally significant fermentation products are SCFAs: butyric, propionic and acetic acid, as well as lactate, which plays a key role in gut health.

Lactic acid ($C_3H_6O_3$) fermentation can be separated in two main pathways. Pathways are homolactic and heterolactic fermentation, where homolactic fermenters directly reduce almost all of their pyruvate to lactate and heterolactic fermenters form substantial amounts of by-products such as ethanol, acetate and carbon dioxide. The two main lactic fermentations differ in complexity as well. Homolactic fermentation [Equation 4] catabolizes glucose via the simple Embden-Meyerhof-Parnas pathway (glycolysis) to pyruvate, which is not decarboxylated as in the respiration but used as H-acceptor. The heterolactic fermentation [Equation 5] on the other hand uses pentose phosphate pathway, which involves an intermediate that is five-carbon xylulose 5-phosphate. The intermediate is

cleaved by phosphoketolase to form glycerin aldehyde phosphate and acetyl phosphate, which will be respectively converted to ethanol and lactate. The homolactic and heterolactic fermentations both use lactate dehydrogenase enzyme in the final step to reduce pyruvic acid to lactic acid. Genera that includes intestinal lactic acid bacteria (LAB) are *Lactobacillus*, *Streptococcus, Enterococcus,* and *Bifidobacterium*. LAB are highly saccharolytic and lack most anabolic pathways. In result, they require very complex nutritional environment as they are insufficient in converting small units to larger molecules for use in reproduction. The configuration of lactic acid that is produced varies among species within genera, thus both D(-) and L(+) enantiomers as well as DL-combination can be formed. (Gottschalk 1986)

$$glucose \xrightarrow{homofermentative\ pathway} 2\ lactate \tag{4}$$

$$glucose \xrightarrow{pentose\ phosphate\ pathway} lactate + ethanol + CO_2 \tag{5}$$

Propionic acid ($C_3H_6O_2$) fermentation uses lactate or glucose as a substrate. Many bacteria convert glucose to a mixture of propionate, acetate and CO_2 [Equation 6]. Several propionate-fermentative bacteria are also able to utilize lactate. Such phyla like Bacteroidetes and Firmicutes produce propionate as major end-product, however as for substrates the Bacteroidetes utilize polysaccharides, whereas the Firmicutes can use organic acids like lactate. (Reichardt 2014). For lactate-utilizing fermentation there are two pathways; the acrylate pathway and the succinate-propionate pathway. The acrylate pathway occurs only in a few microbes such as Megasphaera elsdenii and Clostridium propionicum. For the acrylate pathway any chiral configurations of lactate may be used as the isomerase enzyme racemase is present and it will interconvert enantiomers. Three moles of lactate will produce two moles of propionate and one mole of acetate along with some carbon dioxide and water [Equation 7]. (Gottschalk 1986) The major lactate utilizing propionate pathway is the succinate-propionate pathway [Equation 8].The succinate acts as an intermediate in the fermentation. However, some bacteria can ferment succinate as their major end-product and resulting product can be utilized for production of propionate by another bacteria. (Scheifinger & Wolin 1973). Genera with gastrointestinal significance and propionate production abilities are not limited to, but include genera of *Veillonella*, *Clostridium* and *Selemonas.*

$$1{,}5\ glucose \rightarrow 2\ propionate + acetate + CO_2 \quad (6)$$

$$3\ lactate \xrightarrow{acrylate\ pathway} 2\ propionate + acetate + CO_2 + H_2O \quad (7)$$

$$lactate + NADH + H^+ + ADP + P_i \xrightarrow{succinate-propionate\ pathway} propionate + NAD^+ + ATP + 2H_2O \quad (8)$$

Butyric acid ($C_4H_8O_2$) fermentation is generally associated with obligate anaerobes from genera *Clostridium*, *Eubacterium*, *Fusobacterium* and *Butyrivibrio*. Typically, acetic acid is produced as a co-product of butyrate fermentation [Equation 9]. (Moat *et al*. 2002) Ferredoxin is an iron-sulfur protein and plays a key role in the pathway of butyrate formation. To elaborate, the pyruvate that is formed by the typical Embden-Meyerhof-Parnas pathway is decarboxylated by pyruvate-ferredoxin oxidoreductase for use in acetyl CoA formation. Subsequently, several enzyme-catalyzed electron transfers are then carried to form butyryl phosphate, which will lose its phosphate group to ADP and in result will transform to butyrate. Butyrate forming pathway is energy-wise efficient and will yield 3 ATP [Equation 10]. Solvents like butanol and acetone can be produced by butyrate fermenting bacteria as well if exposed to acidic environment. The shift from butyrate to acetone and butanol fermentation in *C. acetobutylicum* occurs when pH drops below 5. (Gottschalk 1986) However, in the large intestine, pH is near neutral and this type of fermentation would not occur in healthy beings.

$$4\ glucose \rightarrow 3\ butyrate + 2\ acetate + 8CO_2 + 8H_2 \quad (9)$$

$$glucose + 3\ ADP + 3P_i \rightarrow butyrate + 2CO_2 + 2H_2 + 3ATP \quad (10)$$

Acetic acid (CH_3COOH) is fermented as a by-product by several bacteria. Most species can form acetate from H_2 and CO_2 [Equation 11] and hexoses can be converted to three moles of acetate. (Gottschalk 1986) Strictly acetic acid producing bacteria i.e. homoacetogens have been isolated from human feces. The fermentation was stimulated by addition of formic acid (Wolin *et al*. 2003).

$$4H_2 + 2CO_2 \rightarrow acetic\ acid + 2H_2O \quad (11)$$

In the mixed acid fermentation, bacteria metabolize pyruvate to a mixture of acids. It is characteristic for genus *Escherichia*, *Salmonella* and *Shigella*, which ferment sugars to lactic, acetic, succinic and formic acids. In addition, ethanol, CO_2 and H_2 are formed. (Gottschalk 1986)

3.3 Microorganism population of GI tract

Contents and density of microbiota vary across the GI-tract. The stomach has high acidity which kills most microorganisms. As a result, the density in human stomach is 10^1 – 10^3 viable microbes per milliliter of gastric fluid. Stomach is populated mainly by *Streptococcus*, *Staphylococcus*, *Lactobacillus* and yeasts such as *Candida* spp. Some mycobacteria are particularly resistant to gastric pH as well (Willey *et al*. 2013). The small intestine, which is major food digestion and adsorption site is divided into three areas: duodenum, jejunum, and ileum. The duodenum, due to close proximity to stomach's acidic juices as well as inhibitive effect of pancreatic secretions and bile, has low bacterial density (10^1 – 10^3 cfu/mL). When moving forward to down to jejunum and ileum, the pH will become neutral to slightly alkaline (Willey *et al*. 2013). As a result, microbiota will develop density (10^4 – 10^7 cfu/mL) and take characteristics of colon microbiota (O'hara & Shanahan 2006). Most dominant bacteria of broiler chicken small intestine are lactobacilli, which represent 80 to 90% of total bacteria, while remainder consists mainly of enterobacteria and enterococci [Figure 4]. Some of the most dominant cecal bacteria are also found in the distal ileum, which might be due to reverse peristalsis (Rinttilä & Apajalahti 2013). Large intestine, particularly cecum in broiler chickens houses most diverse and dense (approximately 10^{11} cfu/mL) microbe population of GI tract (Apajalahti & Kettunen 2006). Microbiota of lower intestine is dominated by obligate anaerobes due to low luminal redox potential (Apajalahti & Kettunen 2006). Bacteria of cecum can be considered as scavengers because nutrients that reach the colon are residues of host's metabolism occurring in small intestine. Most abundant bacteria in chicken cecum that constitutes for 60 % of total cecal microbiota are families *Lachnospiraceae* and *Ruminococcaceae* from the order of *Clostridiales* [Figure 5].

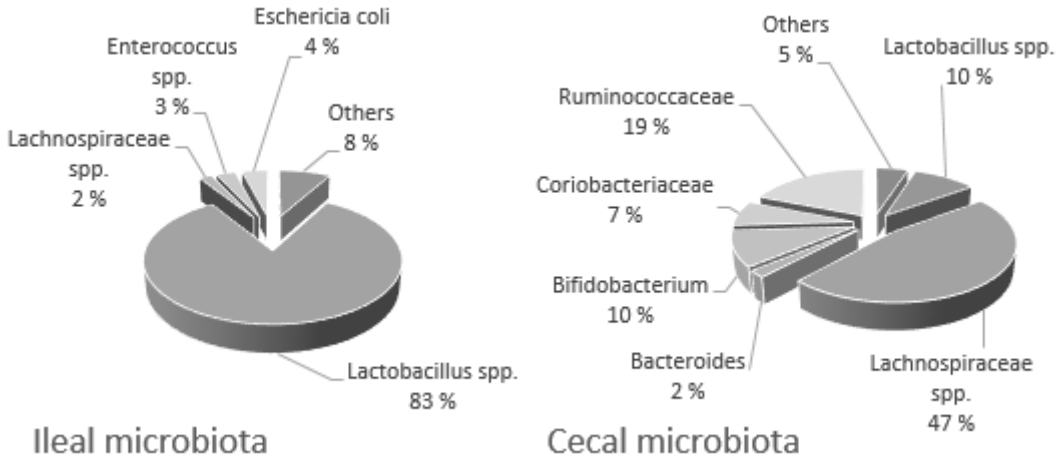

Figure 5 Average microbiota composition (%) in the ileum and cecum of broiler chicken from ten European commercial farms. (Apajalahti & Vienola 2016)

4 Cultivation of obligate anaerobes

Robert Hungate perfected anaerobic cultivation, and several modifications of his technique have been described. Even in present day, three of the essential steps he pioneered are followed: i) use of bicarbonate buffer system ii) use of cysteine as reducing agent and iii) use of resazurin as oxidation-reduction indicator. (Wolfe 2011) In a simplified example, buffered media with reducing agent and redox indicator is prepared under nonsterile conditions. It is made anoxic by boiling and cooling down under carbon dioxide (CO_2) gas flow. Media is aliquoted to gassed (CO_2), hence, O_2-free serum bottles which are then closed with butyl rubber stoppers and crimped with metal seals. Serum bottles are sterilized by autoclaving. All inoculations are carried with a hypodermic syringe and needle (Mil.ler & Wolin 1973).

4.1 Management of anoxic conditions

For successful cultivation of strict anaerobic bacteria, it is required to be conducted not merely in oxygen free media but in highly reducing as well. Highly reducing environment has low redox potential, and by lowering the redox potential further, a desirable decrease in oxygen concentration of the medium will be achieved. Redox potential is a measure of tendency for a chemical substance or ion to acquire electrons and be reduced. Pair of molecules that can be interconverted by the addition or loss of electrons are referred to

as a redox pair. Reducing agent donates electrons to another reactant. Donation will reduce the oxidation state of receiving reactant while the reducing agent will be oxidized (Poole 2015). The commonly used reducing agent is cysteine-HCl (cysteine hydrochloride). Active ingredient of cysteine-HCl is cysteine. However, cysteine with hydrochloride salt possesses high aqueous solubility compared to pure cysteine, 650 g/L vs. 280 g/L respectively (Merck online store). Additionally, when dissolved, due to the acidic hydrochloride, cysteine-HCl results in low pH at which cysteine is stable and insensitive to the oxidation by oxygen. Thus, cysteine-HCl is often preferred to use for preparing stock solutions. In culture media, where pH is adjusted to neutral, thiol, one of the functional groups of cysteine will readily react in presence of oxygen. As a result, two thiols will form a disulfide-bridge between two cysteine residues and will transform them to cystine and water [Figure 6].

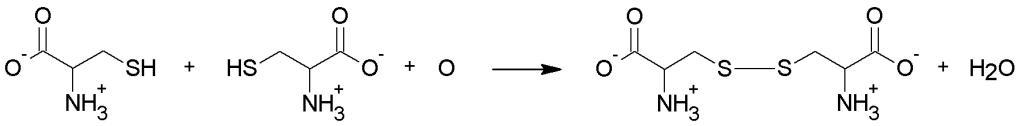

Figure 6 The chemical reaction where two cysteine residues bond together in the presence of oxygen, which will transform them to cystine and water molecules. Binding occurs via disulfide bridge between thiol functional groups.

Redox indicators are widely used for detection of oxygen contamination in anoxic media. Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is typical redox sensitive indicator dye and is suitable for detection of redox potential down to -110 mV. Resazurin undergoes two reactions. First, when boiled, blue resazurin will turn to pink colored resorufin. The first reduction of resazurin is irreversible. The second reduction to dihydroresorufin will make the medium colorless, which is a reversible reaction and will take place at reduction potential of -110 mV. [Figure 7]

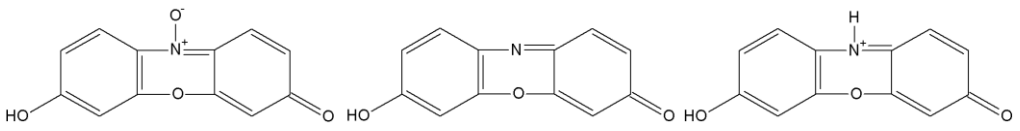

Figure 7 Structures of oxidized, partly reduced and reduced forms of resazurin. Resazurin-N-oxide (purple), resorufin (pink) and dihydroresorufin (colorless) respectivley.

The pink color of the medium does not always imply increase in redox potential due to contamination by oxygen. Certain nitrate reducing microbes produce nitrite (NO_2^-). Nitrite

is a potential oxidant and may increase redox potential above -50 mV, which will turn resazurin to pink. Reduction of nitrite favors anoxic condition where oxygen, a more energetically favorable electron acceptor is not present. Thus, bacteria respire nitrate as substitute terminal electron acceptor. It is to be noted that some organisms require redox potential below -300 mV. Thus, monitoring of anoxic conditions in growth media with resazurin should be carried with a slight precaution. (DSMZ Special Instructions)

The oxygen scrubber is copper-filled column that serves purpose of removing trace oxygen (O_2) from the gas supply. Typical oxygen scrubber consists of large copper tubing, approximately 30 mm in diameter and is filled with copper metal light turnings. Copper metal turnings provide large amount of surface area. Surface of pure copper (Cu) is readily oxidized and the reaction [Equation 12] is catalyzed by higher temperatures such as 325 ℃. The resulting copper(II) oxide (CuO) is darker in color compared to pure copper. The column of oxygen scrubber is wrapped in the flexible heating tape that is suitable for heating metal to high temperatures and is insulated in a container.

$$2\,Cu(s) + O_2(g) \xrightarrow[325\,℃]{} 2CuO(s) \tag{12}$$

Prior to use, the oxygen scrubber must be prepared; copper inside the tubing has to be reduced by running hydrogen gas (H_2) through the column. The contaminant oxygen will be eliminated as water (H_2O) and as a result of the reaction, pure non-oxidized copper will be recovered [Equation 13].

$$CuO(s) + H_2(g) \xrightarrow[325\,℃]{} Cu(s) + H_2O(g) \tag{13}$$

4.2 Nutrients

Complex growth media like peptone is often preferred for bacterial cultures as it includes several different carbon sources to satisfy a wide variety of microbes. However, in this experiment the intension is to use substrates that do not meet requirements of many microbes. Bacteria that can utilize dietary fiber, resistant starch (RS) and soy protein as well as lactic acid are of particular interest. Resistant starch is chosen to mimic the starch that passes regular starch digestion occurring in small intestine. Dietary fiber and RS are both known to act as prebiotics (Topping *et al*. 2003). Wide range of trace elements and

vitamins i.e. micronutrients need to be added artificially for growth since enriching will potentially dilute mutualistic bacteria over time and required cofactors will not be available for enriched bacteria to survive. Cofactors are iron-sulfur clusters or inorganic ions such as metal ions Mg^{2+}, Cu^{+} and Mn^{2+}. Cofactors are not part of enzyme and often their function is to distract substrate's electrons, which will result in an open bonding site for enzyme. Cofactors include complex organic molecules called coenzymes (Hasim & Onn 2010).

4.3 Viability monitoring and composition analyses

Fo the successful execution of this experiment, it is important to monitor the viability and reproduction kinetics. Various metabolites can be associated with digestion of different substrates; in addition, some metabolites are produced regardless of carbon source. Therefore, during the experiment the growth kinetics of bacterial cultures will be monitored and after the experiment metabolites produced will be subject to analysis and in-depth interpretation.

Anaerobic digestion of dietary fiber produces CO_2 as well as volatile fatty acids (VFA). VFA reacts with bicarbonate buffer to release CO_2. Thus, gas production occurs simultaneously and in concert with fiber digestion. Monitoring of gas can provide quantitative information on the rate and efficacy of microbial digestion so it can be used to approximate growth phase. The resulting information can be used to adjust incubation periods (Schofield *et al*. 2014). The pH measurement could potentially be used to monitor activity of acid producing microbes. However, in this experiment, the culture media has very strong buffering capacity and pH monitoring is used solely to confirm that pH of the culture is not approaching values, which are harmful for the proliferation of microbial community. Moreover, pressure changes can be microbicidal. Increased pressure will dissolve the redundant CO_2 into the culture medium from which it can penetrate into bacterial cells and, upon rapid release, eventually disrupt them. Also, carbon dioxide in water solution can form carbonic acid (H_2CO_3) [Equation 14]. High concentrations of CO_2 in culture medium can potentially lower pH and resulting acidity will lead to non-beneficial environment for many bacteria. (Deps-Louka *et al*. 1999)

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \qquad (14)$$

Microscopy can be used to look for morphological changes of bacterial communities. Feeding on specific substrate might gradually affect the bacterial composition profile of cultures. In addition, short-chain fatty acids are of a particular interest and are subject to analysis. The SCFA compositions of enriched cultures are most likely to be unique in regards to substrate preferences of microbial community, and this is a key factor in characterization. Ammonia is formed as a result of proteolysis. Thus, increase of ammonia concentration would indicate increase of proteolytic activity performed by protein-utilizing bacteria. The culture enriched with soy protein concentrate should hypothetically have highest ammonia content. Relevant bacterial families, genera and species associated with animal health and performance will be quantified using real-time quantitative PCR. Total bacterial quantities can be used to approximate the total biomass generated.

5 Materials and methods

The thesis project required considerable amount of manual labor including intricate maneuvers to retain anoxic conditions. Preparations were performed in not the most efficient sequence as availability of biological materials was limited to certain dates. The nature of this study required flexibility and the details of procedure would be alternated if the study would be repeated. All precautions and maneuvers for retaining anoxic conditions cannot be described in complete detail.

A total of seven different substrates were used to enrich otherwise identical cultures. The chosen substrates were: four carbohydrates, protein, lactic acid and fat [Table 1]. Final concentration of all solid substrates in growth medium was 1 g / 100 mL. Concentration of lactic acid was 0.1 M. Carbohydrates are the most significant ingested energy source for bacterial fermentation in cecum. Thus, most of substrates were various oligo-, and polysaccharides such as dietary fiber and starch. The control culture did not include any of the seven substrates. The chosen carbohydrates were primarily of commercial origin. Xylan- and mannan-oligosaccharides were poultry feed additives and the potato starch was a food grade market product. Mix of several fibers consisted of xylan from birch wood (6/10), xylan from oat-spelt (3/10), and soluble wheat fiber (1/10), which was extracted as a part of the innovation project course in Metropolia University of Applied Sciences. The fiber mix is the closest portrayal of naturally occurring fibers in this experiment. Xylan-oligo saccharide is a manufactured product and besides oligosaccharide content (approximately 30 m-%), the test product is known to include glucose. Mannan-

oligosaccharide is a complex produced from cell wall of yeast and depending of manufacturing process has a variable protein and lipid content. The used soy protein concentrate had a purity of 64 %. Fiber and lipid content was approximately 5 m-% each. The choice of substrate for the representing lipids was food grade vegetable oil. The control medium without any of fore mentioned substrates was inoculated as well. Substrates are presented in Table 1 for quick reference.

Table 1 Short description of all used substrates.

vegetable oil	food grade oil from grocery store
fiber mix	commercial (9/10) and extracted (1/10)
lactic acid	commercial reagent
mannan-oligosaccharide	glucomannoprotein-complex from yeast cell wall
potato starch	food grade starch with resistant properties
soy protein concentrate	soy bean-based feed additive
xylan-oligosaccharide	xylose polymers with prebiotic properties

5.1 Preparations

The simplified flow chart of growth medium preparation is presented in Figure 8.

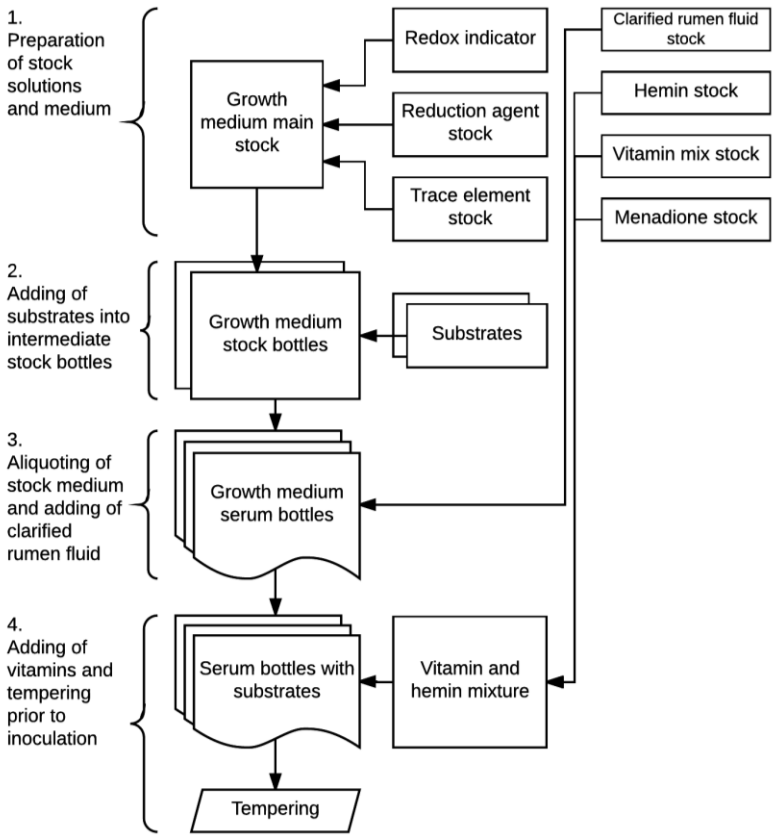

Figure 8 Flow chart displaying the phases of growth medium preparation.

The equipment for retaining anoxic conditions during preparations is displayed in figure 9. The glove box that is appearing on the left was not used for preparations.

Figure 9 Equipment used for utilizing the Hungate technique included: the oxygen scrubber on the top shelf, the gas containers on the right, and tubing attached to the adapter that splits the gas into eight output lines. Attached to the tip of each rubber tube are curved gassing needles, as seen in the circle-shaped close-up. These needles are used for filling the serum bottle with shielding gas, which replaces the oxygen. (Ülle 2017)

5.1.1 Reduction agent

First, the reducing agent was prepared as portions. For the amount of reducing agent needed for two liters of complete stock media, the following ingredients in exact order were added: 0.625 g of cysteine to gassed (CO_2) serum bottle (100 mL). Anoxic de-ionized water (95 mL) was poured on to the added cysteine. The pH of solution was adjusted with 1 N NaOH (4 mL). Finally, Na_2S x 9 H2O was added (0.625 mL). The bottle was closed with butyl rubber stopper and crimped with metal sealing.

5.1.2 Trace element solution

The mineral-containing trace element solution was prepared on magnetic stirrer under CO_2 flow (1.25 mL/min) in 1 liter storage bottle. The first step was to dissolve nitrilotriacetic acid (3 g) into anoxic and de-ionized water (1 L). Subsequently, pH was adjusted to 6.5 using saturated potassium hydroxide (KOH). All the minerals [Table 2] were added and final pH was adjusted to 7 using saturated KOH.

Table 2 Concentrations of minerals per liter of growth media.

$MgSO_4 \times 7\ H_2O$	0.09	mMol
$MnSO_4 \times H_2O$	0.02	mMol
NaCl	0.15	mMol
$FeSO_4 \times 7\ H_2O$	2.9	µMol
$CoSO_4 \times 7\ H_2O$	5.1	µMol
$CaCl_2 \times 2\ H_2O$	5.5	µMol
$ZnSO_4 \times 7\ H_2O$	5.0	µMol
$CuSO_4 \times 5\ H_2O$	0.3	µMol
$KAl(SO_4)2 \times 12\ H_2O$	0.3	µMol
H_3BO_3	1.3	µMol
$Na_2MoO_4 \times 2\ H_2O$	0.3	µMol
$NiCl_2 \times 6\ H_2O$	1.0	µMol
$Na_2SeO_3 \times 5\ H_2O$	9.2	µMol
$Na_2WO_4 \times 2\ H_2O$	9.8	µMol

5.1.3 Growth medium

A ten liters' stock of growth medium were prepared on two separate days, five liters each. For five liters of stock medium, five liters of de-ionized water with 50 ml of trace element solution and buffering reagents with resazurin was made anoxic by boiling in a five liter flask, which was stationed on heating mantle (Barnstead EM5000) and secured to a stand. Meanwhile, oxygen scrubber was heated to 325 °C and reduced by hydrogen mix gas (H_2 5% + N_2). Flowrate of gas mix was adjusted to 5 L / min and reducing of copper was carried for 15 min. After reducing of copper, CO_2 gas was switched on and subsequently nitrogen mix was turned off. The flowrate of carbon dioxide was adjusted to 5 L / min and two out of eight cannulas were set to gas the five liter flask. The heating mantle was shut down and boiling flask was securely moved and stationed on ice to cool down while it was gassed with carbon dioxide. After cooling down, two and a half portions of reducing agent solution and 49 g of sodium bicarbonate ($NaHCO_3$) were added into flask and contents were stirred using a magnetic stirrer. The resulting stock media was aliquoted (500 ml each) to gassed (CO_2) one liter bottles. Substrates were added to aliquot bottles, which were then closed with butyl rubber stoppers and crimped with metal seals.

Lactic acid substrate was neutralized to pH 7 with KOH prior to inclusion. All stock media was refrigerated. Later on, the media with various substrates was further aliquoted (35 mL each) to 100 mL serum bottles and the bottles were refrigerated.

5.1.4 Clarified rumen fluid

Clarified rumen fluid was prepared by centrifugation and autoclaving. A rumen-fistulated cow donated two liters of rumen fluid for this thesis project. The rumen fluid was transported in pre-heated thermos vessels to laboratory within two hours of sampling and screened through steel wire mesh into gassed (CO_2, 1.25 mL/min) five liter flask. Six polypropylene centrifuge bottles (Nalgene) of 500 mL size were gassed for 15 minutes (0.625 mL / min of CO_2 each) prior to filling with 450 mL of rumen fluid. Rumen fluid was maneuvered using 25 mL serological pipette and controller. The work was carried rapidly to prevent oxygen contamination through plastic centrifuge bottles and pipette. Filled centrifuge bottles were centrifuged at 18 500 g for 15 minutes. Supernatants of each bottle were pooled carefully to gassed (CO_2, 1.25 mL/L) five liter flask and aliquoted (100 mL each) to gassed (CO_2, 0.625 mL/L) 100 mL serum bottles, which were closed with butyl rubber stoppers and crimped with metal seals. Anoxic serum bottles containing centrifuged rumen fluid were autoclaved at 121 °C for 20 min to terminate microbial activity.

Prior to any use of syringe to transport fluids between bottles, the following precautions were taken to minimize the risk of contaminating bottles with bacteria or oxygen: the cap surface of butyl rubber stoppers were sprayed with ethanol (70 V-%) and the syringes were purged with CO_2 and flushed with anoxic de-ionized water.

The autoclaved and cooled down rumen fluid was divided by injection into substrate-containing growth media bottles (4 mL in each) using syringe (BD, 5 mL, luer lock tip). Culture media bottles were autoclaved at 121 °C for 20 min. An injection of clarified rumen fluid into sealed serum bottle caused negative pressure, which is noted in gas production calculations. Single bottle containing potato starch as a substrate was opened for redox potential measurement. The redox potential was -400 mV.

5.1.5 Vitamin and hemin stock solutions

The vitamin mix stock solution was prepared in one liter gassed (CO_2, 1.25 mL/L) serum bottle by diluting the ingredients [Table 3] into anoxic de-ionized water. The solution was aliquoted by filter sterilization (Whatman, 0.2 µm) to 100 mL serum bottles. Serum bottles were closed with butyl rubber stoppers and crimped with metal seals. Vitamin mix aliquots were covered with aluminum foil to prevent exposure to light. Vitamin mix stock solution aliquots were refrigerated.

Table 3 Concentrations of vitamins per liter of growth media.

biotin	0.07	µmol
folic acid	0.04	µmol
pyridoxine-HCl	0.4	µmol
thiamine-HCl	0.1	µmol
biboflavin	0.1	µmol
nicotinic acid	0.3	µmol
D-Ca-pantothenate	0.2	µmol
vitamin B12	0.6	nanomol
p-aminobenzoic acid	0.3	µmol
lipoic acid	0.2	µmol

The hemin stock solution was prepared by weighting 100 mg of hemin into 2 mL Eppendorff-tube and adding 2 mL of 1 N NaOH. The resulting solution was translocated to 200 mL volumetric flask, which was filled to the mark with de-ionized water. The hemin stock solution was refrigerated.

The menadione (vitamin K_3) stock solution was prepared by dissolving 50 mg of crystalline menadione in 10 mL of 95 % ethanol. One milliliter of the menadione solution was injected into a sealed and sterile anoxic 100 mL serum bottle filled with de-ionized water. The menadione stock solution was refrigerated.

The vitamin mix, hemin and menadione stock solutions, 30 mL each were combined by injecting them into a single gassed (CO_2, 1.25 mL/L) serum bottle. The stock solution combination (vitamin mix, hemin, menadione; 1:1:1) was then injected into the substrate- and clarified rumen fluid-containing serum bottles (1.2 mL each). The injection was carried in such manner that there were no pressure changes created in the growth media bottles. After every discharge of the solution into the growth bottles, the volume (1.2 mL), was replaced with anoxic CO_2 gas from the gaseous phase of the bottle that was han-

dled. The gas was then translocated by injection into the stock solution combination containing bottle (vitamin mix, hemin, menadione; 1:1:1) to neutralize negative pressure created by initiative charge.

5.2 The experiment

5.2.1 Inoculation

Ten broiler chickens were sacrificed and their digesta-containing caeca were prepared and sealed into plastic bag, which was transported in an anaerobic case to the laboratory within three hours after sampling. In the laboratory, the anaerobic chamber was gassed (N_2) and sterilized instruments such as scissors, spatulas and a beaker as well as a digital scale and serum bottles containing the growth media were moved in. Upon arrival, the sample material was moved into the anaerobic chamber where the digesta was emptied from the cecum into the beaker. The digesta was homogenized using a spatula and weighed (approximately 4 g each) into the growth media bottles. Weighing was carried directly into opened serum bottles with the help of a syringe (BD, 5 mL, luer lock tip) without a needle tip. Bottles were closed with butyl rubber stoppers and moved out of anaerobic chamber. On the desk near the anaerobic chamber each bottle was crimped with metal seal. Inoculated culture bottles were moved into the incubation room and placed on the gyratory shaker. Temperature near the shaker was adjusted to 37 °C.

5.2.2 Gas production measurements

Measurements of gas production volumes were performed manually using Samco glass syringes. Syringes used were in two sizes: 20 ml (without luer lock) and 50 ml (with luer lock). Luer lock fitting needles attached to the tip of syringes were 0.6 x 25 mm in size and their make was Terumo Neolus 23G. All gas production measurements of a single time point were carried with a single needle. The tip of the needle was inserted through the butyl rubber stopper in to the gaseous phase of serum bottle without making contact with fluid; thus, not cross-contaminating. Measurements took place in the incubation room where ambient temperature was 36 °C. Image which displays the experiment setup in the incubation room a moment before the gas measurement can be seen in figure 10.

Figure 10 Several serum bottles containing cultures are placed on top of the gyratory shaker. Glass syringe and hypodermic needles were used for gas measurements. Walls of the incubation room are thermally insulated. (Ülle 2017)

5.2.3 Enrichment procedure

Half an hour before the inoculation, the stock solution combination (vitamin mix, hemin, menadione; 1:1:1) was injected (1.2 mL) into inoculum-free growth media bottles and they were moved into the incubation room and stationed on shaker for tempering. Final gas production volumes were measured in the end of each growth cycle. Further inoculation was carried by transferring 4.4 mL of fluid from each culture into respective tempered bottles containing sterile growth media. Injection with syringe (BD, 5 mL, luer lock tip) causes positive pressure into the bottles, which is noted in gas production calculations. Samples of the cultures at the end of each growth cycle for further analysis were taken after propagation. The enrichment was performed for 8 consecutive days. The incubation times for the first five cycles were 20 hours and for the final cycles 24 hours.

5.2.4 Sampling and analyses

Multiple replicates of final cultures from the end of the last cycle were preserved. Similar basic principles are applied to storage of aerobic as well as anaerobic microorganisms as full recovery of vital functions after preservation was a necessity. Cryopreservation at sub -70 °C in glycerol is the most used method. It has been shown that anaerobes can be stored for long periods in glycerol (Bryukhanov & Netrusov 2006). Four replicates of all isolates (2 mL each) were stored at -80 °C in 10 mL serum bottles with 37% glycerol (1 mL).

All samples were subjected to quantification of relevant intestinal bacteria. The microbial DNA was extracted using in-house method and 16S rRNA was targeted for amplification. SCFA concentrations in the samples were analyzed with in-house gas chromatography method in two runs. Ammonia concentrations were also determined using the colorimetric indophenol-based in-house method.

6 Results

Results are categorized by method or analyte. A fair amount of results will not be presented due to non-significant importance. For example, first enrichment cycles (1 – 5) will be omitted from the SCFA results. During the first propagations bacteria were in a shock state due to rapid change in the environment and collected data is of no significance. Most of the presented results are from the final enrichment cycle. Complete data regarding gas production kinetics will not be presented. This is due to sole purpose of measurements, which was to approximate health and growth rate of gas-producing microbial communities. The choice of food grade vegetable oil for a substrate was a hasty decision and all of the measurements are similar to the control culture. Thus, results of vegetable-oil-enriched cultures will not be presented. Better results might have been achieved if simpler substrates like monoglycerides were used. Lipids are fairly unfamiliar in the field of bacterial fermentation and there has been more interest for substrates like dietary fibers and resistant starch as they provide evident health benefits.

6.1 Growth monitoring

The gas production volume measurements of final (9th) enrichment cycle are presented in Figure 11. The complete gas production graph that displays measured volumes at the end of every enrichment cycle is presented in the Appendix 1.

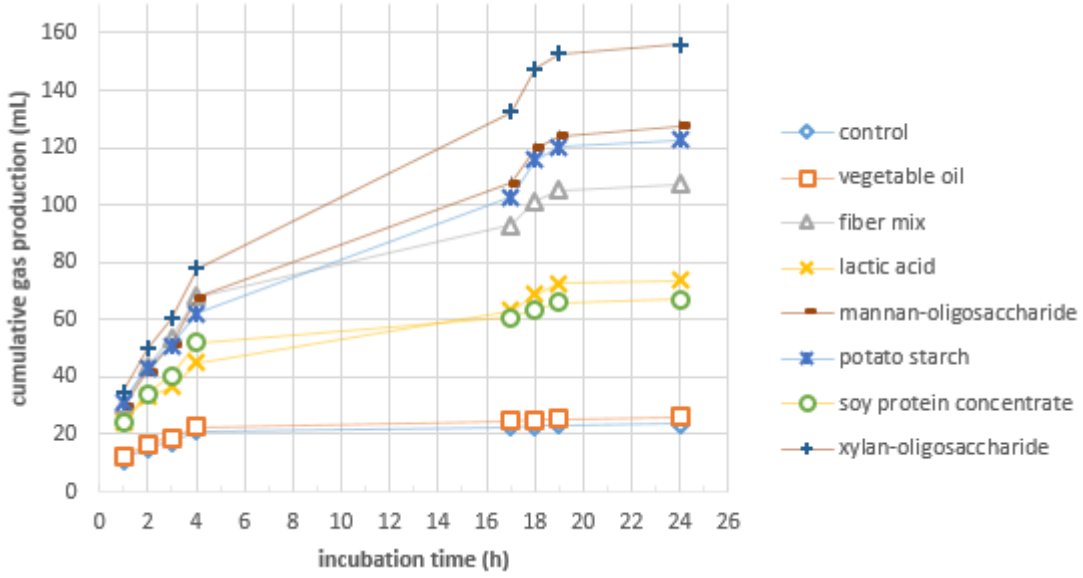

Figure 11 Gas production graph of final enrichment cycle. Symbols indicate the gas measurement time point. Connecting lines are for clarifying purposes only and do not indicate actual measured volume.

6.2 Short-chain fatty acid production

Three major end-product fatty acids of bacterial fermentation (butyrate, propionate and acetate) are presented in Figure 12. SCFAs concentrations that were initially present in growth media as well as metabolite fatty acids that were formed during incubation and transferred in inoculation at the end of each enrichment cycle, are subtracted from the raw data.

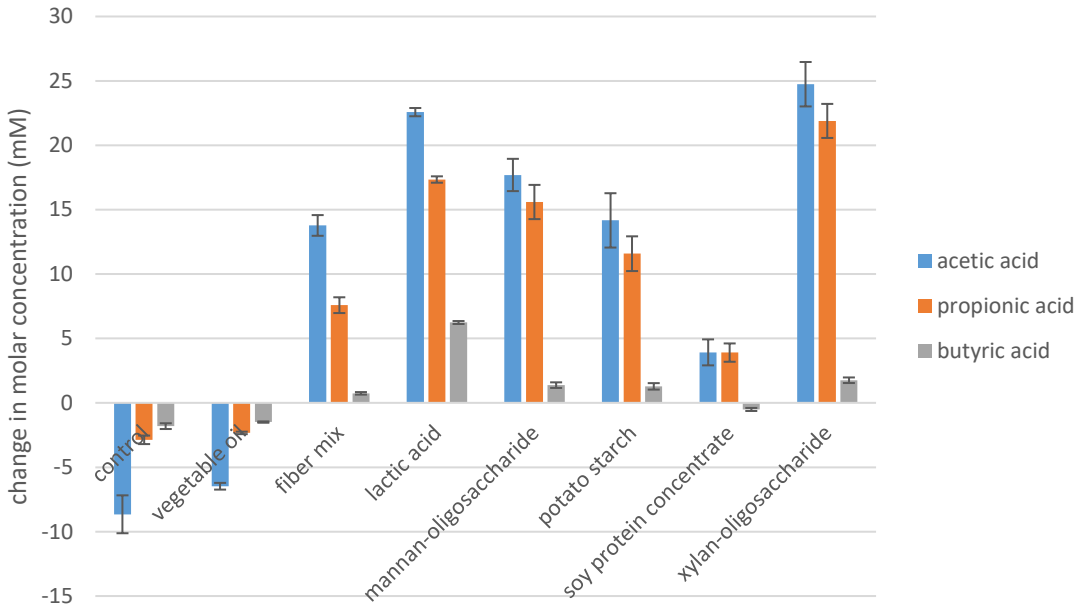

Figure 12 Concentrations of major SCFAs at the end of final enrichment cycle. Error bars indicate standard error of the mean.

SCFA and lactic acid concentration kinetics of lactate-enriched-culture from the four final enrichment cycles are presented in Figure 13.

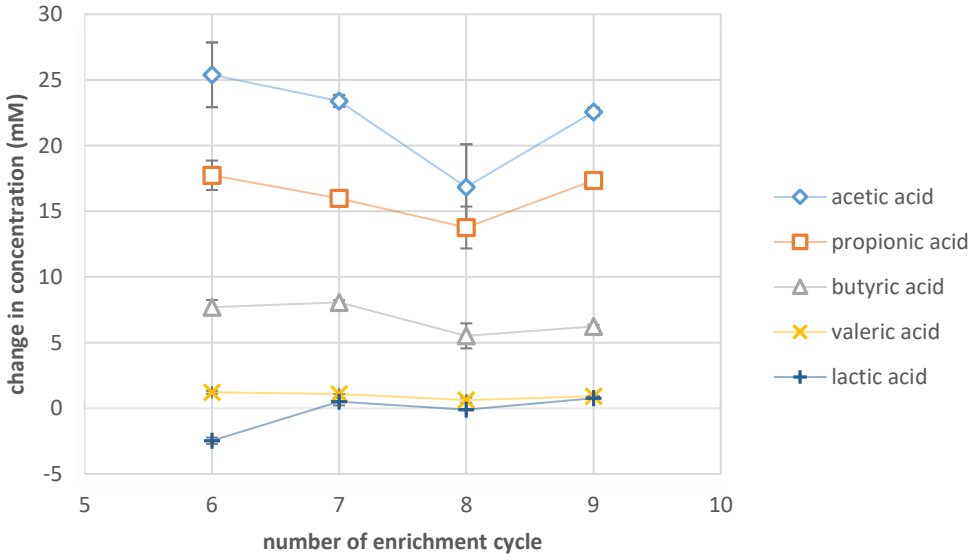

Figure 13 Acid concentrations in lactate-enriched culture at the end of final enrichment cycles (6, 7, 8 and 9). Positive values indicate produced fatty acids while negative values indicate consumed fatty acids. Error bars represent standard error of the mean.

6.3 Microbiota composition

Several bacterial families, genera and species that are known to inhabit the broiler chicken cecum were quantified from enriched cultures by qPCR. The leftmost column in Figure 14 represents the distribution of bacteria in non-incubated control sample (Time point 0), while the other columns represent the distribution of bacteria in enriched cultures. Complete bacterial distribution kinetics are presented in Appendix 2.

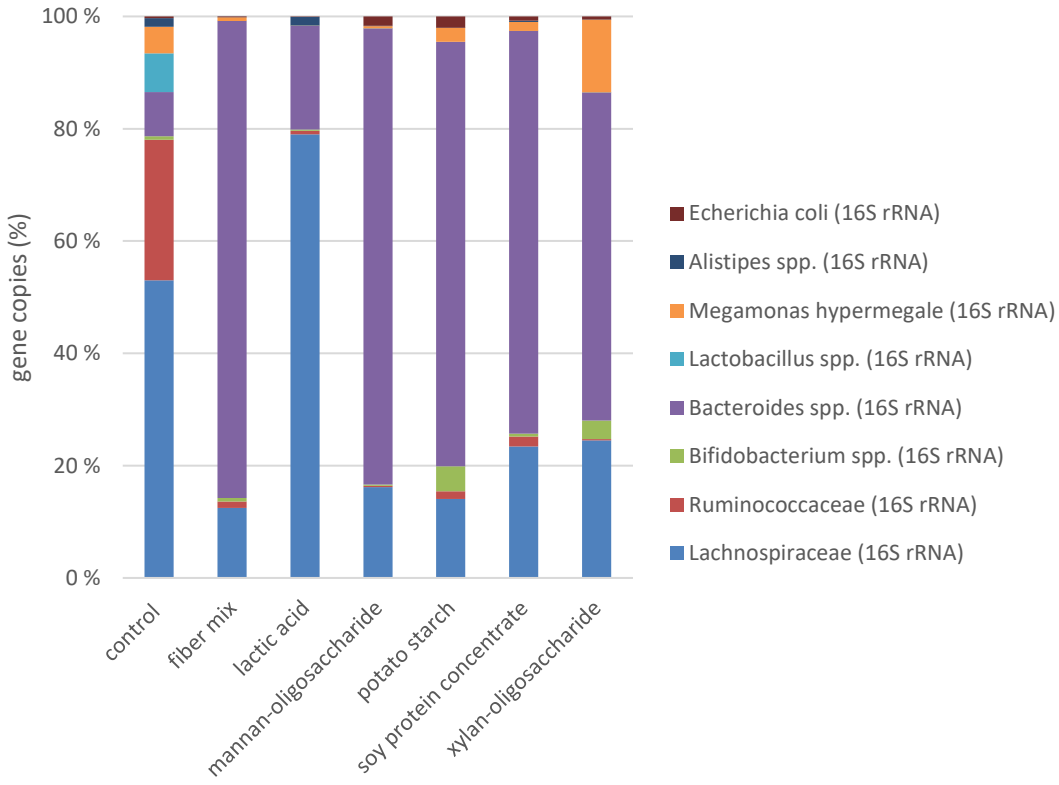

Figure 14 Distribution of intestinally significant bacteria in enriched cultures. Control column illustrates the state of culture without added substrate, 15 minutes after initial inoculation. Other columns represent the state of enriched cultures at 9th and final cycle after 24 hours of incubation.

The total bacteria counts would be traditionally determined by plate counting. However, this technique is time consuming and particularly in the case of obligate anaerobes practically impossible. Therefore, real-time quantitative PCR analysis of total bacterial numbers was applied to compare bacterial reproduction outcome in the presence of different substrates. It is important to understand that results of qPCR method will include also dead bacteria, whereas plate counting provides information of only bacteria that are capable of reproduction. There is also a category of viable but not culturable bacteria that undergo hibernation; thus, they are not readily cultured (Heim *et al*. 2002).

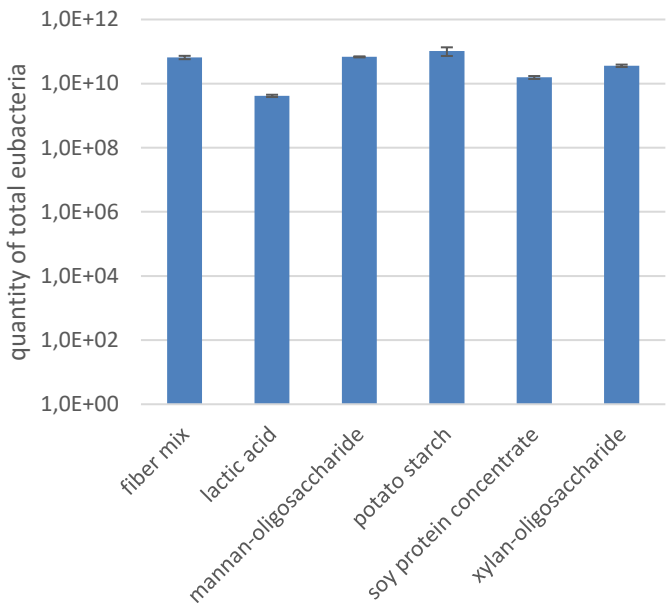

Figure 13 Quantities of total eubacteria. This can be viewed as an approximation of total biomass. Error bars indicate standard error of the mean. Quantities were determined by qPCR from microbial DNA extracted by in-house method of Alimetrics Ltd.

6.4 Ammonia concentrations

The Figure 16 presents the ammonium concentrations at the end of final enrichment cycle in contrast to initially available ammonia that was included in growth media.

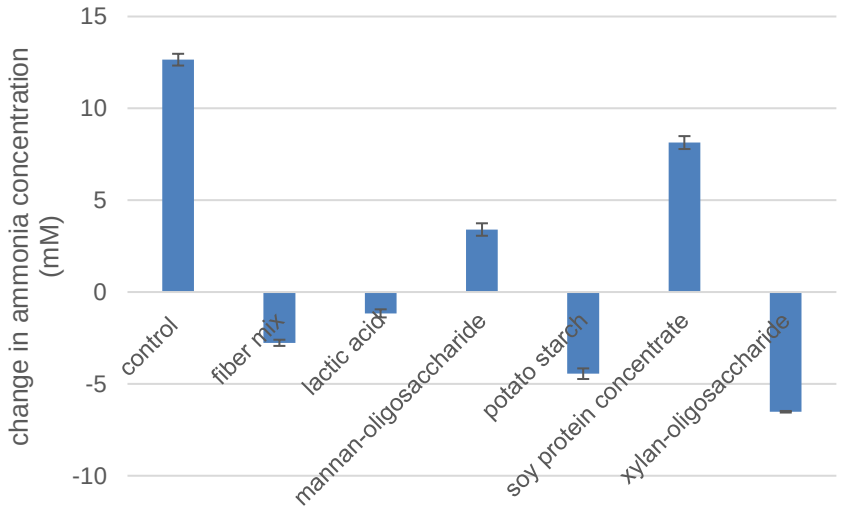

Figure 16 Changes in ammonia concentration. Control column represents the ammonia concentration of culture without added substrate, 15 minutes after initial inoculation. Other columns represent the ammonia concentrations of enriched cultures at 9th cycle after 24 hours of incubation. Error bars indicate the standard error of the mean.

6.5 Factor analysis

Factor analysis was performed in order to reduce the amount of data and explore correlations. The factor analysis was completed using SPSS. The extraction method was principal component analysis and three factors were forced to be extracted. Often the number of extractable factors is based on eigenvalue. The three factors are assumed to represent the three major substrate groups (carbohydrates, protein and lactic acid). Factor analysis rotation method was set to direct oblimin as it was observed from metabolite and bacterial composition analysis data that extracted factors might potentially correlate. All resulting coefficients below 0.3 limit were omitted from the results as they are generally considered non-significant. The analyzed variables included concentrations of all analyzed metabolites as well as gene copy count of amplified bacterial species and genera. Gas production could not be included in the analysis as it lowered the KMO value (Kaiser-Meyer-Olkin measure of sampling adequacy) below the accepted threshold. The data of four final propagation cycles was chosen to be included in the factor analysis. The resulting KMO value was 0.583. Values that are at least 0.5 – 0.6 are considered acceptable which means that the data is suitable for factor analysis.

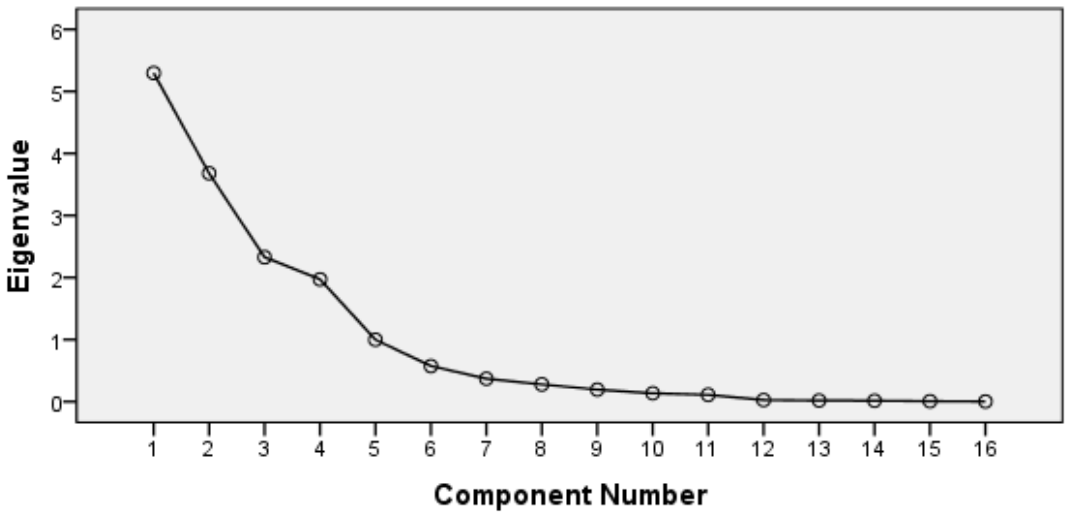

Figure 14 Scree plot of factor analysis. It can be seen that four components are above the critical limit (Eigenvlue 1). However, there is a noticeable shoulder in between components three and five.

The three components that were extracted and their respective loadings are presented in Table 4.

Table 4. Results of factor analysis. The pattern matrix includes component columns 1, 2, and 3 that represent extracted components. The coefficients inside the columns indicate positive or negative loadings. The method used is principal component extraction with oblimin rotation and Kaiser normalisation.

	Component		
	1	2	3
acetic acid	-,795	,304	
propionic acid	-,524		
butyric acid	-,526	,795	
lactic acid		,847	
Lachnospiraceae			,717
Ruminococcaceae		-,367	,532
Bifidobacterium spp.	-,358	-,618	
Bacteroides spp.		-,493	,611
Lactobacillus spp.			,776
Megamonas hypermegale		-,528	-,492
Alistipes finegoldii	,852		
Echerichia coli			,882
valeric acid		,977	
isobutyric acid	,808		
2-me-butyric acid	,827		
isovaleric acid	,957		
ammonium	,782	,390	

6.6 Morphology of bacterial cells

Morphological analysis was conducted on cultures that were stored in formaldehyde. Microscopy images are presented in Appendix 3. The microscope used was Carl Zeiss Axio Imager with 100x objective and immersion oil.

7 Discussion

7.1 Growth monitoring

During the beginning of this experiment, gas production volume comparison verified that actual substrate-specific metabolism of bacteria was occurring. The control culture (without added substrate) was lowest in gas production compared to all other cultures. Excessive microbial acid formation and subsequent uncontrolled pH drop in the vessels was initially considered a threat for success of the experiment, as lower intestinal bacteria typically produce SCFAs as their metabolites as discussed above. However, the lowest measured pH value even after 35 hours of incubation was an acceptable 6.4 (xylan-oligosaccharide). Thus, pH monitoring was not carried further. The typical incubation duration of each enrichment cycle was 20 to 24 hours. The selected incubation duration was a compromise, since different intestinal microorganisms have individual growth kinetics. It was observed that too long a gap between gas production measurements might have resulted in high pressure inside the culture bottles, which potentially inhibited growth of microbes.

The gas production measurements revealed that after sustained incubation period, gas production of all cultures was temporarily reduced. Observed inhibition and restoration of gas production was most likely due to a high cumulated amount of gas. The formed gas was forced to dissolve into the culture medium as a result of increased pressure. Thus, when measuring gas production, a part of gas was still dissolved in the liquid medium. After neutralizing the pressure, the excessive dissolved gas was readily released into the atmosphere of vial, which was added to the gas produced before the next measurement. The pressure might also affect the growth rate of microbes. However, some cultures produced significantly lower amounts of gas and pressure changes cannot explain irregular kinetics for all substrates. Handling of culture vials during the gas measurements included manual vigorous shaking; thus enhancing exposure of sedimented non-soluble substrate particles (fiber mix and soy protein concentrate particularly) for microbes. Additionally, formation of pH-gradient could be regarded as a potential inhibitory factor if culture vials would have been stationed completely still. In this case, the acidic metabolites of bacteria would suspend near the insoluble substrates, making the environment non-optimal for growth. However, in this study the inhibition as a result of pH-gradient was unlikely the reason, since the culture vials were placed on a gyratory

shaker during incubation. Therefore, it can be concluded that irregular kinetics of gas production was caused by the combination of prolonged incubation periods without sufficient shaking and inconsistent intervals of gas measurements.

7.2 Short-chain fatty acid production

The SCFA concentration balance of control and vegetable oil enriched cultures was negative, which indicates that microbes in these cultures utilized more acids than what was produced. Xylan-oligosaccharide and lactic acid cultures contained the highest concentrations of total short-chain fatty acids, while the smallest concentration was observed with a soy protein concentrate. The results also showed that lactate-utilizing bacteria produced the highest amount of butyrate. The amount of produced butyrate in lactate-enriched culture was almost 4-fold when compared to xylan-oligosaccharide, which produced the second highest amount of butyrate. It has been shown that health-promoting butyrate is often produced as a metabolite of bacteria capable of utilizing lactate. However, information about the lactate-utilizing microorganisms present in broiler chicken cecum is lacking. It is assumed that in the large intestine of pigs and rodents the main bacterium responsible for utilization of lactate is *Megasphaera elsdenii*. This species produces propionate, butyrate, acetate and valerate as metabolic end-products (Duncan *et al*. 2004). As presented in Figure 9, all the major SCFAs were produced by lactate-enriched microbial culture.

The precise amount of added lactic acid as a substrate was 100 mM. The total concentration of produced SCFAs is approximately 50 mM. With this information, it can be approximated that half of lactic acid was used for biomass and carbon dioxide generation, as it is not transformed to other acids.

7.3 Microbiota composition

The bacterial profile of control sample resembles the results of Alimetrics Ltd laboratory, in which the average bacterial composition of the broiler chicken cecum from 10 different European commercial farms was evaluated (Apajalahti & Vienola 2016). The results of this thesis project showed that the genus *Bacteroides* was abundant in all except lactate-enriched cultures. *Bacteroides* can process complex biological polymers to simpler ones

which assists neighboring bacteria. It can also break down a large variety of glycosidic bonds. The main energy sources for many species of *Bacteroides* are simple monosaccharides as well as polysaccharides, and more specifically glycans (Wexler 2007). Glycans can be polysaccharides or carbohydrate portions of glycoconjugates (Dwek 1996). Thus, the fermentation that is carried out by *Bacteroides* can use glycoproteins as their substrate source. This explains the abundance of *Bacteroides* in the soy-protein-enriched culture. The soy protein concentrate was only 63 m-% pure and had 5 m-% dietary fiber content which most likely occurred as glycoconjugates of protein. Low fiber-to-protein ratio would not be an issue in the abovementioned hypothesis as the protein was likely to have greater molecular weight in contrast to conjugate fibers (which would be lightweight oligosaccharides).

All substrates with the exception of lactate were not pure and included various carbohydrates including simple sugars. Due to this fact, it is difficult to draw any conclusions about the effect that various substrates might have on biomass generation. However, it can be seen that total eubacteria counts for all substrates are within 100-fold range [Figure 11]. A low number of total generated biomass by using lactate as a substrate can be explained by purity. Lactic acid was the only pure substrate in this experiment; thus, it was very selective. Moreover, lactic acid fermentation produces less ATP; thus, less biomass is generated. Total eubacteria qPCR primers applied in intestinal microbiota research are known to not cover all intestinal microbes and it is subject for discussion among researchers. For example, in this experiment the genus *Bacteroides* surpassed the quantities of total eubacteria in all but lactate-enriched cultures. The forementioned result could also be explained by efficacy of standard amplification.

7.4 Ammonia concentrations

As expected, final concentrations of ammonia were the highest in protein-enriched culture. Moreover, an interesting observation was that mannan-oligosaccharide (MOS) -enriched culture had a positive ammonia concentration balance as well. This is due to the fact that MOS is a glycoprotein complex so it does include also protein to some extent. Carbohydrate-enriched cultures utilized initially available ammonia from the growth media. This indicates that microbe populations synthesized amino acids from ammonium content for use in growth. It is likely that the breakdown of dead microbes i.e. putrefaction

was occurring in every culture at some level. This should result in an increase of ammonium concentration. However, in carbohydrate-enriched cultures, the utilization of ammonium for synthesis of amino acids exceeded the formulation of ammonium that formulated from metabolism of putrefying bacteria.

7.5 Factor analysis

The scree plot suggested that it should be possible to extract even four factors as the eigenvalue of up to four components is above 1. However, it can be noted that there is a noticeable shoulder in the plot occurring in between components number three and five. This is considered as a weakness for the analysis if four factors were to be extracted.

Component 1 displays a strong positive loading of BCFAs as well as ammonium. Thus, Component 1 comprises characteristics of the culture that was enriched using a protein substrate. The negative loading of *Bifidobacterium* spp. can be rationalized as the particular genus is known to ferment carbohydrates. However, the role of *A. finegolii* is unclear. Component 2 shows strong positive loading between concentrations of butyric and lactic acid as well as valeric acid. It is known that lactate-utilizing bacteria often ferment butyrate as their major end-product. From the results, it can be seen that *Bifidobacterium* spp. does not play significant role in lactate-to-butyrate fermentation as it has relatively strong negative loading (-0.62). Component 3 has strong loading of bacterial species that are associated with utilization of various carbohydrates. The negative loading of *M. hypermegale* could indicate that the bacteria is of no communal significance for carbohydrate-utilizing bacterial communities.

7.6 Morphology of bacterial cells

As expected, the control culture comprised most diverse content in a morphological sense. Cocci, streptococci and bacilli were abundant. Some diplococci and diplobacilli can be found as well. Lactate-utilizing culture produced less total bacteria; thus, microscopy image content is less dense. It can be seen that bacilli utilize lactate. Mannan-oligosaccharide, fiber mix and soy protein enrichments seemed to comprise mainly cocci; however, the bacterial composition analysis showed that *Bacteroides* (bacilli) should be abundant. This is most probably due to the fact that microscopy image shows the non-

soluble particles of used substrates. The culture enriched with the potato starch comprises of diplobacilli and -cocci; however, single-unit variants of fore mentioned shapes are abundant. Within xylan-oligosaccharide enrichment, the class bacilli is abundant with traces of cocci.

8 Conclusions

The enrichment of anaerobic bacterial communities was successful and metabolite analyses could be used to verify that. Typical patterns were observed from the results and explanations for the unexpected or surprising discoveries could be found. It is worth noting that that there would have been room for improvement in the procedure. The growth medium composition was potentially favoring certain bacteria. The overwhelming occurrence of *Bacteroides* was indeed a surprise and even if it was explained, it might indicate a deficiency in the medium. There is a probability that the growth medium did not provide suitable environment for the proliferation of *Lachnospiraceae* which is known to be vastly abundant in cecum of broiler chicken. However, it is most probable that other bacteria outcompeted *Lachnospiraceae* due to composition of used substrates.

The substrates that were supposed to replicate the dietary residues that bypass the small intestine were mainly of commercial origin. It is known that commercially manufactured substrates include a variety of additional content such as simple sugars that do not usually enter in cecum. Simple substrates can potentially offer a growth advantage for some competing bacteria, which may not necessarily belong to the dominant cecal microbiota *in vivo*.

To make the enrichment-isolation method more robust, it would be important to develop a sample preparation procedure, which would mimic the digestion occurring in the small intestine. Utilization of commercial pancreatic and selective brush border enzymes of small intestine could be used to prepare naturally occurring cleaving sites for cecal bacteria. Filtration techniques such as ultrafiltration could be used to separate the simple molecules that result from enzymatic treatment. Therefore, further research and development is required.

Enrichment technique applied in this study could potentially be used to isolate previously non-culturable bacteria. Coupling the enrichment method with gel filtration could be used

to study the effect of substrate's molecular weight on bacterial composition. The key problem in enrichment-isolation technique of this study is the insufficient knowledge as well as replication of characteristics regarding nutrients before and after they pass the small intestine.

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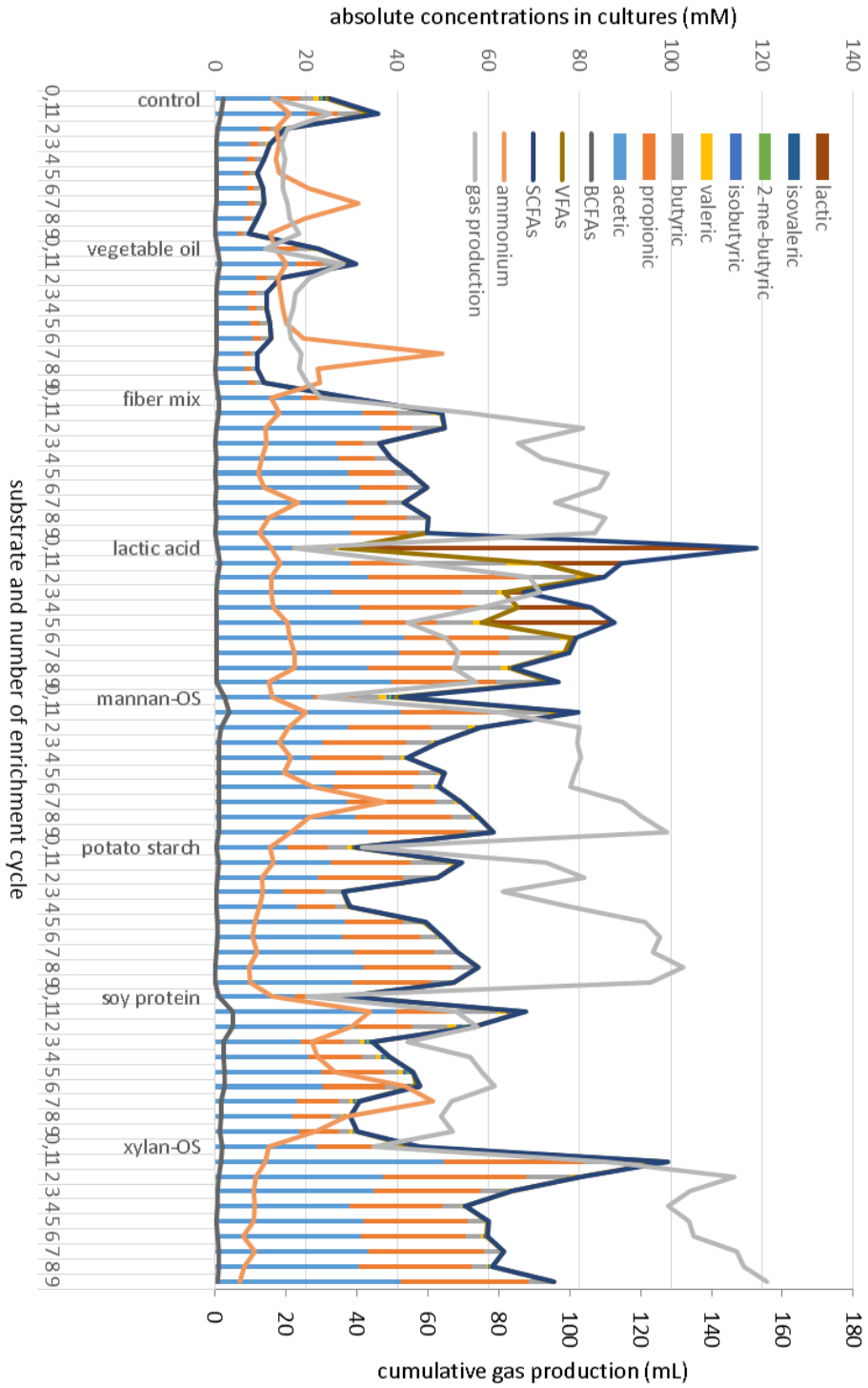
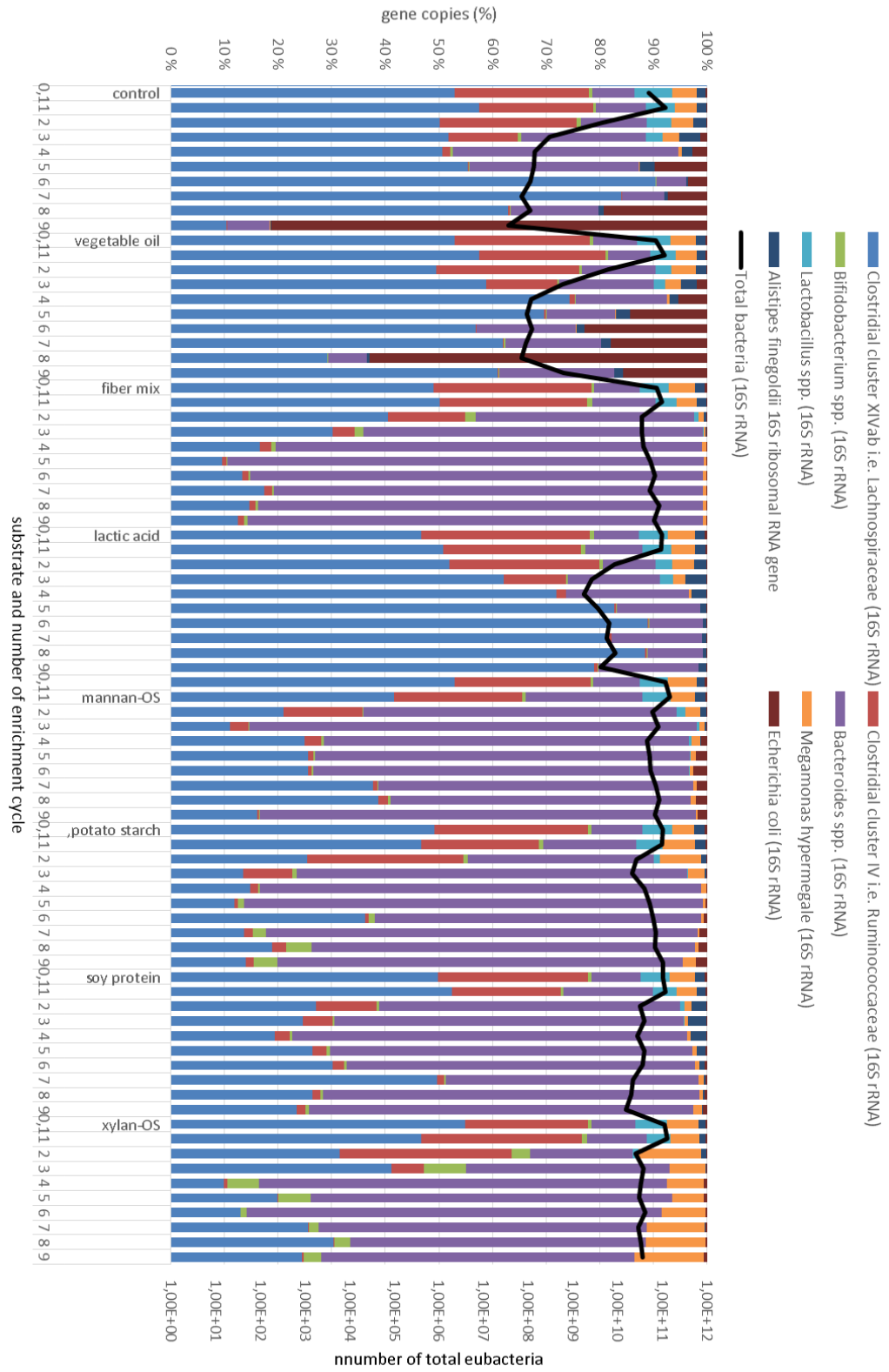
Appendix 1. Combined results

Fatty acid, ammonium and gas production results in the function of enrichment cycle number.



Appendix 2. Kinetics of change in microbiota

Bacterial composition in the function of enrichment cycle number.



Appendix 3. Microscopy images

1000x zoom of culutures that were diluted by a factor of ½ for storage in formaldehyde.