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Carbohydrate utilization and short chain fatty acid production in Bifidobacteria

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Abstract

In the first stage of life infants go through rapid growth and development and have high nutritional needs. During this first 6 months of life breast milk is considered to provide a complete postnatal nutrition. Positive benefits of breastmilk have been extensively documented and it is a known fact that breastfed infants show a domination of Bifidobacterial species in the gut. The colonization pattern has been revealed to be majorly affected by human milk oligosaccharides (HMOs) found in breast milk. HMOs are not digested by the infant and will as a result reach the colon where different strains of Bifidobacteria metabolise them and produce metabolites, mainly in the form of short chain fatty acids (SCFA) where some might be beneficial to the host.

The study included seven different Bifidobacteria strains that were test grown in five different mediums with different carbon sources with a total of 42 samples repeated in three parallels. DNA quantification was performed with the use of qPCR and short chain fatty acid profiles were measured with the use of high-performance liquid chromatography.

In conclusion the results demonstrated differences in HMO utilization and SCFA production among the different strains. It was clearly demonstrated that *Bifidobacterium longum subsp. Infantis* has the best utilization and growth abilities on HMOs which may reflect their abundance in the infant gut.
The main metabolite of carbohydrate fermentation performed by Bifidobacteria strain was acetic acid and the results also confirmed that the most common infant type Bifidobacteria strains are the main producers of lactic acid.

Sammendrag

I den første fasen av livet går spedbarn igjennom rask vekst og utvikling og har dermed et høyt ernæringsbehov. I de første 6 leve månedene anses morsmelk til å være en fullverdig næringskilde for spedbarnet. Det har blitt omfattende dokumentert at morsmelk gir positive helseutfall og at brysternærte barn har en dominerende populasjon av Bifidobakterie arter i tarmen hvor denne spesifikke koloniseringsprosessen har vist seg å være i stor grad påvirket av oligosakkarider funnet i morsmelk. Melke oligosakkaridene er ufordøyelige for spedbarnet og vil dermed ende i tykktarmen hvor de kan bli metabolisert av ulike Bifidobakterielle stammer. Deres metabolitter er hovedsakelig i form av kort kjedede fettsyrer der noen kan være gunstige for verten.

Studien inkluderte syv forskjellige Bifidobakterie-stammer som ble testet i fem forskjellige medier tilsatt ulike karbon kilder. Det var totalt 42 prøver gjentatt i tre paralleller. DNA-kvantifisering ble utført ved bruk av qPCR og kortkjedede fettsyreprofiler ble målt med høyytelsesvæskekromatografi.

Resultatene demonstrerte forskjeller i oligosakkarid utnyttelse samt kort kjeda fettsyre produksjon av de ulike stammene. Resultatene viste at *Bifidobacterium longum* subsp. *Infantis* hadde best utnyttelses og vekstevne av oligasakkaridene, resultatet kan dermed trolig gjenspeile stammes overflod i tarmen hos spedbarn. Hoved metabolitten etter oligosakkarid fermentering var vist å være eddikksyre og resultatene bekreftet også at de vanligste stammene funnet hos spedbarn er de samme stammene som er de viktigste produsentene av melkesyre.

Abbrevations

DNA	-	Deoxyribonucleic acid
dNTP	-	Nucleoside triphosphate
dsDNA	-	Double stranded DNA
Fuc	-	Fucose
Gal	-	Galactose
Gal-1-P	-	α-Galactose1-phosphate
GIT	-	Gastro intestinal tract
Glc	-	Glucose
GlcNAc	-	N-acetyl glucosamine
GLNBP	-	GNB/LNB phosphorylase
GNB	-	Galacto-N-biose
HMO	-	Human milk oligosaccharide
HDAC	-	Histone deacetylase
HPLC	-	High performance liquid chromatography
IL	-	Interleukin
LacNAc	-	N-acetyllactosamine
LNB	-	Lacto-N-biose
LNnT	-	Lacto-N-neotetraose
LNFP1	-	Lacto-N-fucopentaose
LNT	-	Lacto-N-tetraose
LUB	-	L actic-utilizing and butyrate-producing bacterium
MRS	-	De Man Rogosa and Sharpe broth
Neu5Ac	-	Sialic acid-N-acetyl-neuraminic acid
NFκβ	-	Nuclear factor κB
qPCR	-	Quantitative polymerase chain reaction
SCFA	-	Short chain fatty acids
2'FL	-	2'-Fucosyllactose (Blood group O/H antigen triaose type 5)
3'SL	-	3'Sialyllactose (GM3 ganglioside oligosaccharide)

Table of content

1.0 Introduction

Infants have high nutritional requirements and their rapid growth and development is unique in the first stage of life and breast milk is considered to provide the infant a complete postnatal nutrition for the first six months of life. With its complex composition it delivers numerous nutritional compounds for growth and maturation of the offspring precisely during the period which infants develop their innate immunity and gut microbiota. Because breast milk is the sole nourishment for the new-born infant it must by necessity be a complete food to cover the nutritional needs (Smilowitz et al., 2014, Thomson, Medina and Garrido, 2018). Positive benefits of breast milk have been extensively documented showing breastfed infants have lower risk of acute ear infection, asthma (in young children), atopic dermatitis, childhood leukaemia, sudden infant death syndrome in term babies, type 1 and 2 diabetes and necrotizing enterocolitis (NEC) in preterm babies (Pokusaeva, Fitzgerald and van Sinderen, 2011). Reports have shown that breastfed infants have a dominating population of Bifidobacterial species in their gut compared with formula fed infants. The microbial population of the gut have shown that differences in the colonization process can be correlated with the onset of disease later in life or to a protective physiological role, where specifically human milk oligosaccharides (HMO) have been linked as a major determent of the outcome (Milani et al., 2017).

1.1 Breastmilk composition

Breast milk is composed primarily of protein, lipids and carbohydrates, but it also contains vitamins and minerals to support adequate growth and development. Breast milk also contains growth factors, stem cells and anti-microbial components supporting the immature immunity of the infants (Ballard and Morrow, 2013, Dessì et al., 2018). The basic structure is composed of lactose which is the major and most important nutritionally compound. Second most important compound is lipids whereas HMOs are the third most abundant molecular species in term of concentration (Marcobal et al., 2010). The macronutrient composition in breast milk is quite conserved across populations while the oligosaccharide composition varies greatly both between and within women (Milani et al., 2017). HMO levels are fluctuating throughout the day and the lactation period, these variations have been found to influence both the immunity and microbiota in the neonate (Smilowitz et al., 2014). HMOs are energetically costly to produce for the mammary gland, yet indigestible by the infant (Smilowitz et al., 2014). These divers indigestible glycans can reach the large intestine where

they can be metabolised by certain strains of microbiota, where the microbiota produces a wide range of metabolites, mainly in the form of short chain fatty acids (SCFA) (Ríos-Covián et al., 2006).

1.2Basic structure of HMOs

All HMOs are composed of five monosaccharides, but they can vary in composition. These five building blocks are Glucose (Glc), Galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid N-acetyl-neuraminic acid (Neu5Ac). Together these units can form around 200 structurally different HMOs. Breastfed infants will therefore not be exposed to the same set of HMOs with respect to total amount and concentration of the highly structurally variable HMOs (Milani et al., 2017).

The biosynthesis of breastmilk starts in the mammary gland where a lactose core is formed. The lactose core is synthesised out of Gal and Glc catalysed by Beta-galactotransferase in the presence of alfa-albumin. HMOs are either categorized into type 1 chain (Lacto-N-biose containing) (LNB) or type 2 chain (N- acetyllactosamine containing) (LacNAc) oligosaccharides, where type 1 oligosaccharides dominate over type 2 (Fushinobu, 2010). All HMOs follow the same basic configuration: a lactose core on the reducing end (with few exceptions) (Smilowitz et al., 2014) which can be further elongated enzymatically either by β1-3 linkages or by β1-6 linkages to a LNB (Galβ1-3GlcNAc, type 1 chain) or by β1-6 linkage to N-acetylactosamine (Galβ1-4GlcNAc-, type 2 chain) (Marcobal et al., 2010, Milani et al., 2017, Yu, Chen and Newburg, 2013). The branched oligosaccharide chain can be elongated by fucose or by sialic acid, by both or neither. The diversity of the HMOs is therefore a combination of the core structure elongation and branching, together with modifications such as fucosylation or sialyation (Milani et al., 2017). The proportion of these different structures have been reported to be from 35-50% fucosylated, 12-14% sialyated and 42-55% non-fucosylated natural HMOs (Smilowitz et al., 2014).

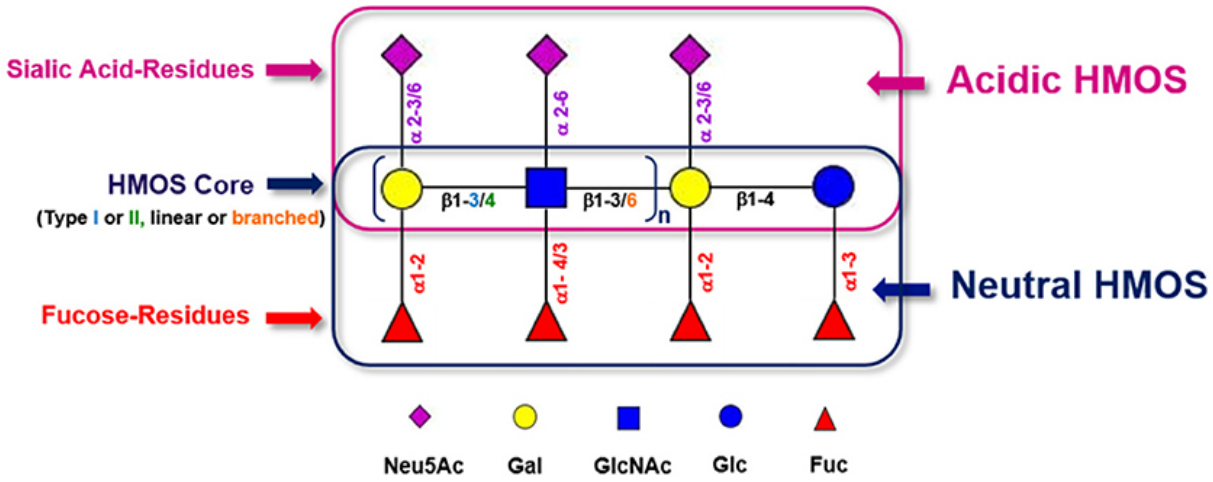

Figure 1. General composition of HMOs. HMOs are either type 1 chain (Lacto-N-Biose containing) or type 2 chains (N-acetyll actosamine) containing chains. The HMO core structure can be extended linear by adding Gal-GlcNAc to the terminal galactoses via either β1-3 glyosidic linkages or β1-6. If elongated by β1-6 glyosidic linkages branching will occur. HMO core structure can be further accessorized with fucose or sialic acid (Ayechu-Muruzabal et al., 2018)

1.3 Genetic determinants of HMO production

Both genetic and environmental factors influence the HMO composition. Although the impact of environmental factors is not fully clear it is well known that variations in fucose and sialic acid composition is dependent on maternal genetics. There are certain genes that can encode for distinct fucosyl transferases and depending on which gene is activated one can express different blood groups, phenotypically described as secretor (Se) status and Lewis (Le) blood group (Smilowitz et al., 2014).

The blood group characteristics are determined by two genetic loci. The Se gene is encoding α1-2-fucosyl transferase (FUT2), while the Le gene is encoding the α1-3/4-fucosyltransferanse (FUT3) (Milani et al., 2017). Women with an active Se locus are categorized as secretors and produce milk which is high in 2'-Fucosyllactose (2'FL), Lacto-N-fucopentaose 1 (LNFP1) and other α1-2- fucosylated HMOs. Women missing an active Se locus are on the other hand categorized as non-secretors and they produce milk with a low concentration of α1-2- fucosylated HMOs. Women with an active Le locus will be categorized as Le positive and these individuals express FUT3 (alfa 1,3/4-fucosyltransferanse) which can transfer FUC by an α1-4 linkage to a subterminal GlcNAc of the type 1 chain (on HMOs). Le-negative categorized women express low levels of these specific α1-4 fucosylated HMOs (Milani et al., 2017 & Smilowitz et al., 2014).

With this as a baseline HMOs can be divided into four groups:

1. Lewis-positive secretors (with active FUT2 and FUT 3)
2. Lewis-negative secretors (with active FUT 2 and inactive FUT3)
3. Lewis-positive non-secretors (with inactive FUT2 and active FUT3) and
4. Lewis-negative non-secretors (with inactive FUT2 and inactive FUT3)
   (Milani et al., 2017)

Even though HMOs are either FUT2 or FUT3 is an all or nothing phenomenon as it is determined by genetics, there are other differential expressions of genes that encode for other components of the cellular glycosylation, which may contribute to the observed variations of HMO composition both between women and throughout the lactation period (Milani et al., 2017).

1.4 Role of HMOs in the establishment on the infant gut microbiota

The human gut microbiota is located in the distal part of the digestive tract and is characterized by a complex microbial community (Matamoros et al., 2013). It is composed of members from Archaea, Eukarya and Bacteria, it might also include some viruses. There has been revealed a mutualistic relationship between humans and the microbiota. The composition of microbiota can vary greatly among individuals both in concentration and in diversity, nonetheless evidence in suggesting some preserved microbial functions in all individuals (Matamoros et al., 2013). The infant gut microbiota is distinctly different from the ones of adults and there are observed even greater interindividual differences. An infant gut is characterized by relative low diversity in both bacterial genera and species but will with maturation increase in diversity and complexity. Somewhere between two to five years of age the microbiota will mature to a more adult and stable microbiota (Milani et al., 2017).

The colonization process of the infant gut is affected by several factors such as mode of delivery (vaginal vs caesarean section) and feeding mode (breastfed vs formula fed) (Milani et al., 2017, Thomson, Medina and Garrido, 2018). When comparing breastfed infants with formula fed, breastfed infants show significantly higher levels of Bifidobacteria and Lactobacillus and lower levels of staphylococci, clostridia, enterococci, *Bacteriocides*, enterobacteria and other pathogenic bacteria, which are all represented at a higher levels in formula fed infants (Harmsen et al., 2000). The characteristic differences between breast and formula fed infants also effects the levels of SCFA. The SCFA levels in breastfed infants

are characterized by high levels of acetate and lactate, lower levels of propionate and low or absent levels of butyrate (Milani et al., 2017).

The general observed trends in the gut of breastfed infants is a domination of Bifidobacteria and Bacteroicides species (Rautava, 2017, Harmsen et al., 2000). The most commonly present human Bifidobacterial species in the gut are *Bifidobacteria breve* (*B.breve*), *B. longum* subsp. *infantis* (*B. infantis*), *B.adolescentis, B. pseudocatenulatum, B. longum* subsp*. Longum, B. dentium* and *B. bifidum* although certain species appear to be more commonly found in the infant gut, such as *B. infantis, B.breve* and *B .bifidum* (Thomson, Medina and Garrido, 2018, Matamoros et al., 2013).

The predominance of Bifidobacteria in breastfed infants is strongly connected to HMOs ability to selectively stimulate and promote growth of Bifidobacteria and therefore also modulate the gut colonization process (Rautava 2017, Smilowitz et al., 2014). This ability is often referred to as the bifidus factor. The bifidus factor is believed to come from the dominance of LNB in HMO structure and due to the presence of GNB/LNB pathway found in infant type Bifidobacteria (Fushinobu, 2010, Kitaoka, 2012). It has also been demonstrated that LNB has especially prebiotic effects in *B. bifidum, B. breve* and *B. infantis* (Fushinobu, 2010).

There are only some of the Bifidobacterial species that can efficiently utilize HMOs as a sole carbon source and clear niche-specific adaptations have been observed in certain strains. In the *B. infantis* lineage there is detected a 43 kb gene cluster solely detected to the transport systems and intracellular glycosyl hydrolases for HMO utilization (Garrido, Barile and Mills, 2012) while species typical for an adult gut (*B. adolescentis, B. catenulatum* and *B. lactis*) lack the genes encoding for HMO metabolism. Other infant type Bifidobacteria strains exhibit specific phenotypic variations (Sela and Mills, 2010) e.g *B. bifidum* exports several enzymes to liberate LNB from the HMO core structure before transporting it into the cell for metabolization, yet other strains as *B. breve* has no capability of HMO cleavage but can utilize liberated monosaccharides from the HMO structure. This kind of phenotypic diversity shows a niche adaptation to a carbohydrate rich gastro intestinal tract (GIT) environment and cross-feeding mechanisms between bacterial strains (Sela and Mills, 2010, Kitaoka, 2012). Cross-feeding is a method of utilizing end products from the metabolism of a given microorganism by another one, but also the utilization of metabolites from complex

carbohydrate degradation by one microorganism performed by another one, such as in the case of *B. breve* (Belenguer et al., 2006, Ríos-Covián et al., 2016). The clear differences in genetic utilization abilities of HMO degradation might explain the domination of *B. infantis* in the infant gut (Pokusaeva, Fitzgerald and van Sinderen, 2011, Mills, 2010).

1.5 Enzymes responsible for the degradation of HMOs

HMOs are complex oligosaccharides that need to be degraded before they can be utilized. Generally polymeric carbohydrates are degraded to low molecular weight oligosaccharides by bacteria. Low molecular weight oligosaccharides can further be degraded to monosaccharides by the use of carbohydrate degrading enzymes and will eventually be converted to SCFA and other organic compounds where some may be beneficial to the host (Pokusaeva, Fitzgerald and van Sinderen, 2011).

Bifidobacterias saccharolytic ability can be confirmed by looking at their genome which encodes for a large number of carbohydrate modifying enzymes. It can also reflect their adaptation to a carbohydrate rich GIT environment. The most common group of enzymes found is the β-galactosidase. β-galactosidase has the ability to synthesize prebiotic substances from lactose which gives Bifidobacteria the ability to grow on both milk and its derivatives such as lactose and other lactose derived galacto-oligosaccharides which contain β-galactosidic linkages. The activity of the β-galactosidase has been found in various Bifidobacterial species, such as *B. adolescentis, B. bifidum, B. longum, B. infantis* and *B. pseudolongum* (Pokusaeva, Fitzgerald and van Sinderen, 2011).

1.5.1 Bifidobacterial enzymes involved in HMO degradation

Glucosidases and other ABC membrane bound transporters are needed for the utilization of oligosaccharides and for the transportation of oligosaccharides to Bifidobacterium's main metabolic pathway (Sela and Mills, 2010, Wada et al., 2008). *B. infantis* secretes extracellular fucosidases for cleavage of terminal fucosyl linkages, permitting for a further degradation of the HMO core. The cleaved HMO structure can be further hydrolysed by different extracellular enzymes in the process of realising LNB as LNB is not found in its free form it must be cleaved and released for degradation from HMO. LNB can be translocated across the cell membrane where an intracellular phosphorylase GNB/LNB phosphorylase (GLNBP) cleaves the GNB/LNB from HMO allowing it to enter the central fructose-6-phosphate phosphoketolase pathway to generate cellular ATP. Bifidobacteria are using this central

fermentation pathway in the metabolism of hexose sugars, which is centred around the key enzyme fructose-6- phosphoketolase, a pathway which is also called the bifid shunt (Sela and Mills, 2010 & Sela et al., 2008, O'Callaghan and van Sinderen, 2016).

1.5.1.1 GNB/LNB pathway

LNB is a building block found in three types of structure 1 HMOs, and galacto-N-biose (GNB) is mostly a core unit in O-glycans in mucin glycoproteins which are present in both milk and human intestine. LNB is bound together with GNB and the cleavage is performed by GLNBP to two separate metabolites which both can enter glycolysis and amino sugar metabolic cycle (Wada et al., 2008). GLNBP is classified as a glycoside hydrolysis and all strains of *B. longum subsp*. *longum*, *B. infantis, B. breve* and *B. bifidum* have been found to possess GLNBP, whereas none of the major strains in adult intestine (*B. adolescentis, B. catenulatum* and *B. lactis)* have been found to possess this glyosidic hydrolysis (Kitaoka, 2012). Studies show that GLNBP may play a vital role in the colonization process of Bifidobacteria in infant intestine (Fushinobu, 2010).

1.6 Metabolites of HMO degradation

The main product after saccharolytic fermentation is SCFA. SCFA are volatile fatty acids and are characterized by containing 1-6 carbon chains existing in straight and branched conformations. The most abundant SCFA in the colon is Acetic acid (C2), followed by propionic acid (C3) and Butyric acid (C4). Acetate, propionate and butyrate are represented in approximately ratio of 60:20:20 in the colon and stool (Besten 2013). The fermentation products from the bifid shunt from Bifidobacteria are mainly acetate and lactate (Lewis et al., 2015). Lactic acid can also be produced by acid bacteria and proteobacteria and while lactic acid is by itself not an SCFA but can further be converted to SCFA by other bacterial species in the gut (Ríos-Covián et al., 2016, Reichardt et al., 2014). Metabolites of HMO degradation are observed to differ when there is excess of carbohydrates and when they are in limitation. Bifidobacteria are mainly producing acetate and lactate when carbohydrates are in excess and mainly acetate and formic acid when there is carbohydrate limitation (LeBlanc et al., 2017). A rapid consumption of an energy source, and mainly consumption of carbohydrates has been shown to result in production of substantial amounts of lactate and low amounts of acetate, ethanol and formic acid. On the other hand, when consumed at a slower rate, less lactate is produced together with increased production of acetate, formic

acid and ethanol (O'Callaghan and van Sinderen, 2016). The total amount of SCFA balance is also majorly affected by bacterial cross-feeding mechanism (Belenguer et al., 2006).

1.6.1 Mechanisms of SCFA production and metabolic routes

The formation of SCFA can occur in different pathways (Reichardt et al., 2014). There is a limited body of literature specifically describing the metabolic pathways in Bifidobacteria.

Acetate and lactate are mostly produced via the bifid shunt by Bifidobacteria as a result of carbohydrate fermentation (Scardovi, 1965, Ríos-Covián et al., 2016). Another major source of acetate formation is produced from glucose carbon skeleton. Propionate and butyrate are also suggested to be produced via the bifid shunt albeit via slightly different routes (Scardovi, 1965). Butyrate is formed out of two molecules of acetyl CoA which together yield acetoacetyl CoA. Via the L (+)-β-hydrohybutyryl Co A and crotonyl CoA, acetoacetyl is converted to butyryl CoA. Butyryl CoA can yield butyrate via butyryl CoA: acetate CoA transferase pathway (Pryde et al., 2002). Majority of the butyrate producers are using acetate CoA-transferase pathway (Flint et al., 2014).

Propionate can be formed via three different pathways and it is not clearly described in the literature which one is applicable for Bifidobacterium although in the propandiol pathway deoxy sugars (such as fucose) are converted to 1,2-propandiol and it might therefore be reasonable to assume that this pathway is used by Bifidobacteria in saccharolytic fermentation (Ríos-Covián et al., 2016).

1.6.2 Potential health effect of HMO metabolites

SCFA levels and type of SCFA have together with other metabolites from gut fermentation been suggested to act as a biomarker for health (Ríos-Covián et al., 2016). SCFA are found in hepatic, portal and peripheral blood showing their diverse area of use. Together with other metabolites they can be taken up by organs and act as substrates or signal molecules (den Besten et al., 2013, Reichardt et al., 2014). SCFA protective role has been of great interest, but research has been mainly focusing on SCFA profiles and health outcomes in the adult population and there is scares available research explicitly investigating SCFA levels in infants and the correlations with health outcomes, even though the infant gut microbiota (and therefore also their SCFA profile) is deviating from the ones of adults. The gut is functioning as an important site of immune education and regulation where the microbiota and their metabolites plays a major role. Alterations or dysbiosis in the gut colonization pattern may

be especially important in infancy and it is therefore suggested that alterations in the colonization pattern may lead to certain immune disorders later in life, as diabetes, allergies and obesity (Bridgman et al., 2017).

There is some conflicting information about the total amount of SCFA in infants and how these levels affect health. Some studies report that exclusive breastfed infants exhibit lower concentrations of total SCFA (which includes acetate, butyrate, propionate, valerate, iso-butyrate and iso-valerate) and show higher concentrations of lactate and acetate. On the contrary formula fed infants show an increase of total SCFA levels in faecal samples which may have metabolic consequences as well as other metabolic risk factors (Bridgman et al., 2017). Other findings suggest that children developing eczema and food allergies exhibit lower levels of SCFA in faecal samples compared with those that do not develop such diseases (Roduit et al., 2018). Research shows conclusive results that breastfed infants have four times higher likelihood of higher acetate and lactate levels opposed to formula fed, and the levels were not connected with external influences such as birth mode, mothers BMI, sex of the baby, antibiotic use during labor or socioeconomic influences (Bridgman et al., 2017).

1.6.3 Acetate

Acetate is after production absorbed by the gut lumen where some is metabolised by the colonocytes, some enters the peripheral circulation to be metabolised by muscle and other tissue, while the rest is taken up by the liver (den Besten et al., 2013, LeBlanc et al., 2017). The utilization of acetate is done in several ways. It can be oxidised in the tricarboxylic acid cycle (TCA cycle) where it is converted to oxaloacetate and take part in the gluconeogenesis (den Besten et al., 2013), it can also be used as a substrate for liver cholesterol and fatty acid synthesis (Pryde et al., 2002, den Besten et al., 2013) and enhance ileal motility by affecting ileal peristaltic`s (Hosseini et al., 2011). Acetate also contributes through cross-feeding mechanism to butyrate production (O'Callaghan and van Sinderen, 2016m, Matsuki et al., 2016, Thomson, Medina and Garrido, 2018).

The elevated levels of acetate in infant gut may play a role in pathogenic protection and has been found to be a key player in the ability of Bifidobacteria to inhibit enteropathogens (Ríos-Covián et al., 2016). Acetate, together with other SCFA, contributes to lowering of the pH levels in the gut lumen as most of the SCFA are absorbed into the blood in exchange of secretion of bicarbonate to the lumen. This fact alone may be an important aspect in

inhibition of pathogenic microorganism and could be particularly important in infants with an immature immune system (den Besten et al., 2013). Infants have impaired pro-inflammatory cytokine production to prevent adverse immunological reactions between mother and foetus during pregnancy, but simultaneously make the infant more prone to infectious disease early in life (Martin et al., 2010). Higher acetate levels in infants may also protect against the onset of allergic disease through assisting in the development of oral tolerance (Bridgman et al., 2017, Arrieta et al., 2015) Children with high acetate levels also tend to show lower prevalence of food sensitization and food allergy (Roduit et al., 2018).

1.6.4 Butyrate

Butyrate is important in normal development of colonic epithelial cells. It also functions as a form of fuel for intestinal cells and increase their mucin production which may result in changes of bacterial adhesion. Butyrate is aiding in the general maintenance of the gut barrier function through the improvement of tight junction assembly by activation of AMP-activated protein kinase (AMPK) (Peng et al., 2009). The regulation of tight junctions is an important step in protecting the host from undigested proteins and other xenobiotics entering the blood stream. Infants with an immature gut epithelium may especially benefit from an improvement in the tight junction assembly, and a compromised epithelial integrity has been linked to disease like asthma, allergies and autoimmunity (Roduit et al., 2018). Butyrate has the ability to affect a series of anti-inflammatory effects in the host. Being a non-competitive inhibitor of histone deacetylase butyrate can support the expansion of T-regulatory cells and increase the levels of anti-inflammatory interleukin 10 (IL-10). By keeping histones in a more acetylated state, expression of certain genes are affected, genes involved in cell differentiation, apoptosis, cell cycle arrest for malignant cells and a down regulation of inflammatory cytokines in mucosal cells (as an inhibition of nuclear factor kappa B (NF- κβ), all of which may benefit the immune supressed infant (Roduit et al., 2018, Segain, 2000, Leonel and Alvarez-Leite, 2012).

What is considered a beneficial level of butyrate concentration in infants is not quite clear, as research is presenting conflicting results. Higher levels of butyrate and propionate have been seen in obese children and adults (Payne et al., 2011) while recent study has showed and association between high levels of both butyrate and propionate early in life with a protective role against atopy (Roduit et al., 2018). There has also been observed a significant

association between high levels of butyrate and propionate in faeces of one-year old children which had significantly lower occurrence of atopic sensitization and were also less likely to develop asthma between three to six years of age (Roduit et al., 2018).
There might be some restrictions in the measurement of butyrate levels as it is quickly absorbed and utilized by the colonocytes for the production of ATP. As much as 70% of the energy supply to the colonocytes is provided by butyrate and therefore very little reaches the portal system (Pryde et al., 2002).

1.6.5 Propionate

Levels of propionate have been studied to a lesser extent, but there are shown correlations between reduced levels of propionate producers and children in the risk of developing asthma, atopic sensitization and elevated levels associated with protection against atopy (Arrieta et al., 2015, Roduit et al., 2018) Although excessive propionate levels can be problematic. Propionic acidemia, a genetical inherent disorder may lead to severe health implications and is correlated with mental retardation, mitochondrial dysfunction and other health problems (Khalesi and Garshasbi, 2017, MacFabe, 2012). Excess propionate faecal levels have also been observed in children with autistic spectrum disorder (ASD) together with an increase of known propionate producing bacteria (as *Clostridia* and *Bacteriocides*) (MacFabe, 2015, Wang et al., 2012, Finegold, 2011).
In general, propionic acid has been shown to have anti-lipogenic and cholesterol lowering effects and it also influences weight control and feeding behaviour. In addition, propionate (like butyrate) has been associated with an antiproliferative effect towards colon cancer cells (Hosseini et al., 2011). Propionate is to a lesser extent absorbed by the colonocytes compared with butyrate, but it is a precursor for gluconeogenesis in the liver and it is estimated that 69% of the total glucose production stems from propionates ability of glucose synthesis (den Besten et al., 2013, O'Callaghan and van Sinderen, 2016). Measurement of propionic acid might be a difficult task as the majority is residing within cells, and not much is in the portal venous system.

1.6.6 Lactate

Breastfed infants also exhibit higher concentrations of lactate and succinate, both being intermediates in production of SCFA. Succinate and Lactate can be converted to propionate via the succinate and acrylate pathway respectively. Lactate can also be used in a cross-feeding mechanism by other lactate utilizing bacteria in the SCFA production and is an

important step in preventing lactate accumulation which can have serious health implications, as inducing metabolic acidosis, neurotoxicity and cardiac arrythmia. Even though infants do show higher toleration for higher lactate levels compared with adults, it is still an important mechanism for protection. Lactate is otherwise considered protective in the infant gut (Pham et al., 2016). High concentrations of lactic acid in adult is correlated with e.g increased risk of ulcerative colitis, whereas for infants' lactate (together with acetate) may be an important mechanism of preventing the overgrowth of pH sensitive pathogenic bacteria, such as Enterobacteriaceae and clostridia (Duncan et al., 2009, Sun and O`Riordan, 2013). Lactate has also been reported to maintain a gut barrier function through stimulation of enterocyte proliferation (Bridgman et al., 2017).

1.7 Methods to quantify bacteria and study SCFA

1.7.1 Quantification and detection of bacteria with polymerase chain reaction

Polymerase chain reaction (PCR) is a method widely used in microbial community analysis. The method is used for DNA detection and is highly accurate with a high sensitivity and reproducibility. The qualitative PCR method is a technique used to measure the number of copies of a gene in a community or an environmental sample (Evans et al. 2013). The sample of interest is mixed with reagents that allow an amplification of the fragment of interest which is composed of unique or custom primers, DNA polymerases, buffers and salts and deoxyribose nucleotide triphosphate (Espy et al., 2006). The method is based on three different steps; denaturation, annealing and elongation all performed at different temperatures and in multiple cycles. In the first phase of denaturation the temperature is raised up to 95°C for the double stranded DNA (dsDNA) to be separated. The GC content of the DNA will affect its stability and higher GC content will require higher temperature for denaturation. An opened dsDNA will allow for primers to attach to their complementary sequence (Schochetman, G. et al. 1988). In the annealing phase primers are able to attach themselves to the DNA. Elongation is done by extending the DNA template from the 3'OH-end performed by DNA polymerase. By incorporating complimentary dNTPs DNA fragment of interest to form a new complementary strand of DNA, resulting in an exponential elongation and a large number of copies of the same fragment (Schochetman, G. et al. 1988).

Principles for quantitative PCR (qPCR) are based on the same principles as of qualitative PCR, but in qualitative PCR the product is only detected at its end point giving no information about the initial amount of the targeted nucleotide sequence whereas with the qPCR method the amount of amplified fragment is measured for each PCR cycle (Espy et al., 2006). There are several different detection principles in qPCR. The most widely used is based in intercalating dyes, such as SYBR green and EvaGreen (Espy et al., 2006). The fluorescence dye is able to bind to dsDNA and is then measured after each cycle. The dye becomes fluorescence when it binds to product DNA, thus the fluorescens will increase proportionally with the increasing product DNA (Evans et al. 2013). In other words, the fluorescence is a relative measure of the DNA content in the sample.

As the qPCR plot is generated there is two phases; an exponential phase where reagents are not limited and a non-exponential plateau phase. In the exponential phase the product will double in each cycle, but as the reaction proceeds components will be consumed and eventually one of the components will be limiting. This slows down the reaction resulting in the plateau phase (Espy et al., 2006).

1.7.2 Use of High-performance liquid chromatography to quantify SCFA

The basic principle of high-performance liquid chromatography (HPLC), is a technique where one can separate mixture of substances into their compounds. The separation is based on the basis of molecular structure and molecular composition. The method involves a stationary phase, which can be both solid and a liquid supported on a solid, and a mobile phase, which can be either gas or liquid. The mobile phase flows through the stationary phase and carries the components of the mixture with it. The substances that have the strongest interaction with the stationary phase have longer retention time in the column compared with the substances with weaker interaction, which will cause a separation of the various components. Chromatographic separations can be carried out using a variety of stationary phases, including volatile gas and liquids (Grushka and Grinberg, 2012).
In the HPLC method the solvent (which goes through the column) is forced through under high pressure of up to 400 atmospheres (Grushka and Grinberg, 2012).

1.8 Aim of the study

There is recently shown great variations in HMO composition across mothers. These differences are especially related to the level of fucosylation. It is well known that HMOs are

selectively promoting growth of Bifidobacteria in the infant gut. The aim of the study is therefore to experimentally determine how oligosaccharides in mothers' milk can affect the SCFA profiles from infant derived Bifidobacteria.

Several sub steps were included in order to achieve the main goal and are listed below.

1. Pilot study was performed were bacteria revival was performed and growth conditions and incubation lengths were tested.
2. Bacteria strains were transferred to several carbon sources and DNA quantification and SCFA profiles were tested.
3. Main project included two more carbon sources with fucosylated and sialyated HMOs.
4. SCFA profiling was performed with HPLC at the biorefinery Lab.
5. SCFA levels were calculated

An outline of the workflow in this study is illustrated in figure 2.
The workflow consisted of bacteria revival and transfer to different carbon sources in anaerobic chamber, DNA purification and quantification and SCFA profiling with HPLC at the biorefinery Lab with guidance from Leszek Michalak.

2.0 Materials and Method

A pilot study was conducted prior to the main experiment. A schematic view of the experiments conducted in the pilot experiment and main experiment is shown in figure 2.

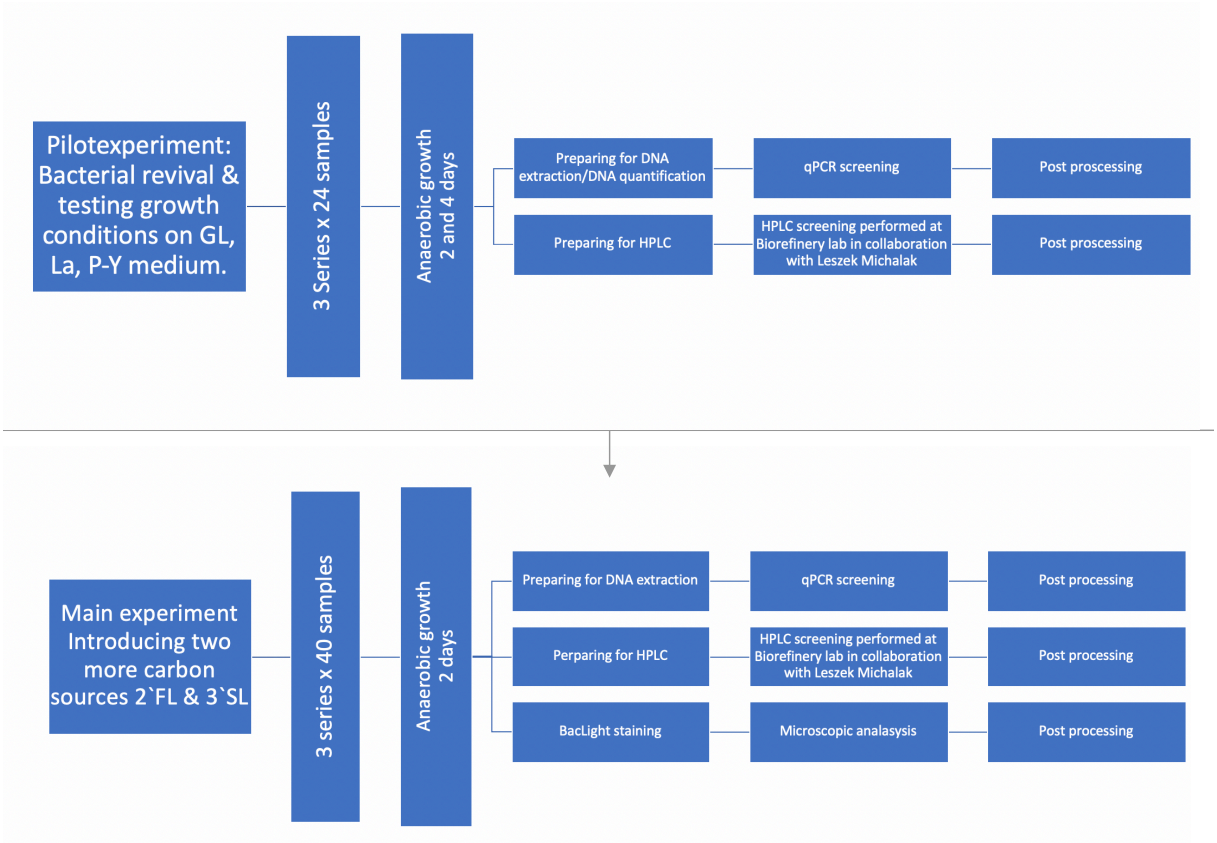

Figure 2: Flowchart of the experimental set up. Two experiments were performed in total, where one of them were pilot small-scale experiments, conducted before the main experiment. The main experiment was based on the results obtained in the pilot study.

2.1 Pilot study, Revival of the bacteria and Bacterial growth medium

Prior to the main study a pilot experiment was conducted. The pilot study was set up to test growth conditions in liquid and solid De Man Rogosa and Sharpe broth (MRS) (Sigma Aldrich) medium supplemented with L-cysteine and Agar powder to the solid medium, to test length of incubation in both anaerobic jar and anaerobic chamber and SCFA profile measurements were tested. MRS is the medium of choice for industrial quality control for the growth of Bifidobacteria. The preparations for growth medium was done following manufacturer's instructions. For detailed description see appendix A. All mediums were autoclaved and stored at 4°C before bacteria cultures were applied. Seven strains of Bifidobacteria were used in total in this project; *Bifidobacterium adolescentis* (DSM 20083), *B. pseudocatenulatum* (DSM 20438), *B. breve* (DSM 20213), *B. longum* subsp. *longum* (DSM 20219), *B. dentium* (DSM 20436), *B. bifidum* (DSM 20456) and *B. infantis* (DSM 20088) (Leibniz-Institut, DSMZ-Deutesche Sammlung von Mikroorganismen und Zellkulturen GmbH). Bacteria strains came in ampoules with dried bacteria culture and protocol given by the manufacturer for the revival was followed. The dried cultures were resuspended in 50 µl of liquid MRS broth, left for 30 minutes and 30 µl of revived bacteria was transferred to agar plates and seeded out and in the liquid broth and solid medium, which had 24 h prior to the seeding been incubated in anaerobic jars at room temperature. Liquid broth was used as a backup as Bifidobacteria strains grow better in liquid mediums than on solidified plates. The plates and tubes with bacteria were incubated for 48 h and 96 h at 37 °C in incubation chamber (Whitley A95 TG). For purity control, one colony from each plate was picked and seeded out on new MRS plates and again incubated for 48 h at 37 °C. Rest of the bacteria colonies were transferred to cryo-tubes with 15% glycerol (Mast group Ltd) and stored at -80 degrees.

Using a sterile inoculation loop one colony from each strain was transferred to Eppendorf tubes with 500 µl Peptone-Yeast extract medium (P-Y) where the colonies were resuspended. Peptone-yeast extract is recommended for the cultivation and biochemical identification of anaerobic microorganism. From the resuspension of bacteria colonies 50 µl was transferred to 3 different mediums; clean P-Y medium, P-Y medium with 10% Glucose solution and P-Y medium with 10% lactose solution. Detailed description of the mediums is given in appendix B.

After 48 h, a sample of 200µl was taken out and frozen at -20°C, the same was repeated after a total of 96 h of incubation time. All samples were then tested on qPCR and based on the CT levels it was decided to proceed with an incubation time of 48 h for the main project. For SCFA profiling the supernatant after 48 h and 96 h was initially tested on gas chromatography (GC) but were excluded from the main study as the GC protocol used in the MiDiv lab were not able to give clear results of SCFA profiles. It was therefore decided to continue with liquid gas chromatography (HPLC) at biorefinery lab. After running some test samples with several different dilution factors a 5times dilution was chosen for the most accurate measurements of SCFA.

2.2 Transfer of bacteria to carbon sources with added HMO

After the pilot study protocols were made and followed for the rest of the project. The same procedures were followed for making of the growth medium and incubation time. In figure 3 is a representation of the work flow during the main experiment.

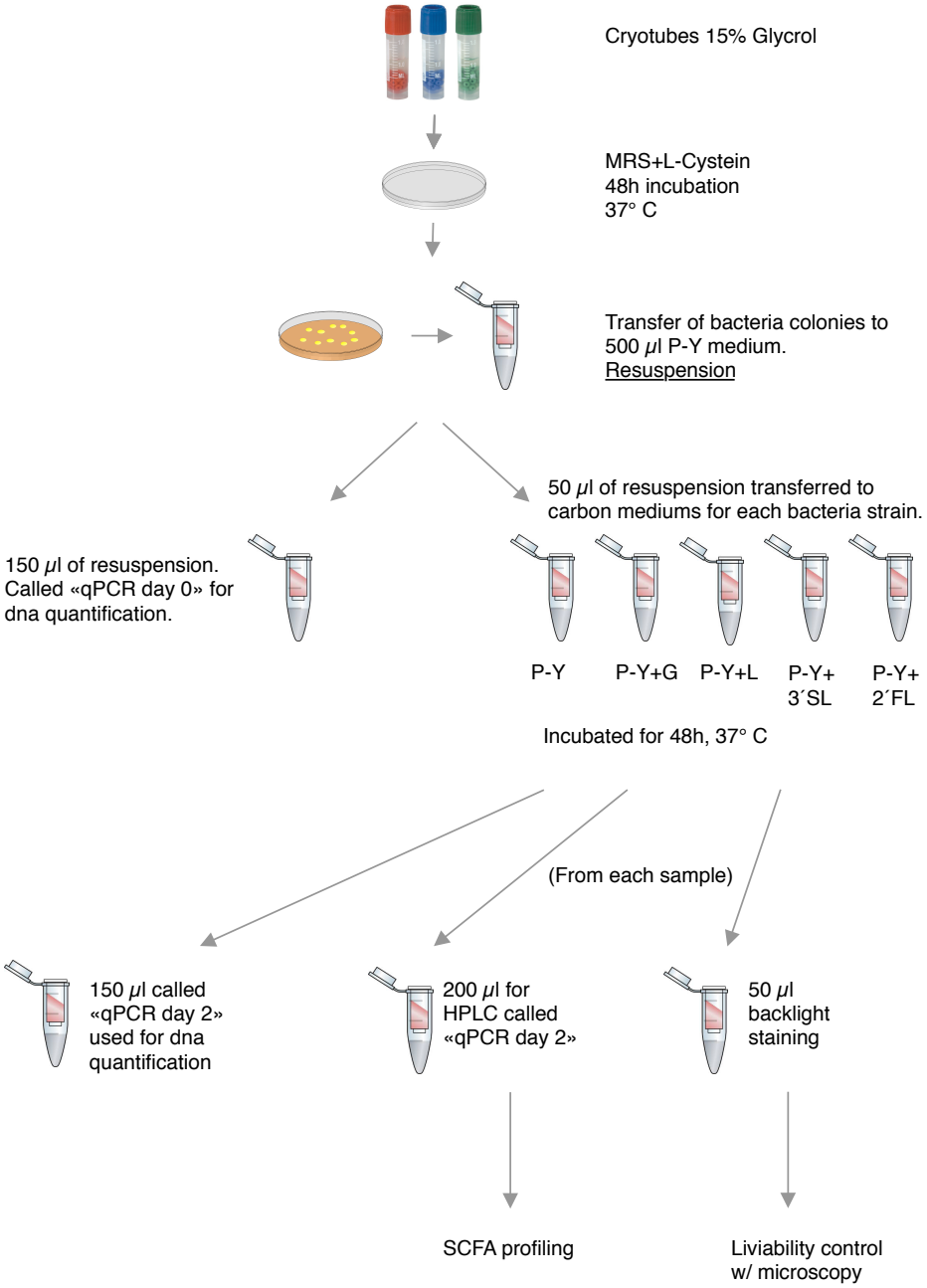

Figure 3 represents the steps followed in the main experiment.

Bacteria strains were transferred from cryo-tubes to agar plates and after incubation transferred to 500 µl P-Y mediums added different carbon sources. For the main project there was added two types of HMO. P-Y medium with 10% N-Acetylneuraminoyllactose (3'SL) and P-Y medium with 10% 2'Fucosyllactose (2'FL) (Elicityl, Oligotech). There was a total of 40 samples, 5 mediums for each strain and one negative control. From the resuspension 150 µl was taken out and frozen at -20°C. This sample was called "qPCR day 0" and was later used for DNA quantification. The remaining samples were incubated in the anaerobic chamber for 48 h. The timespan and concentrations were chosen according to the result conducted from the pilot study. After the 48 h 200 µl was transferred from sample to new Eppendorf tubes and frozen immediately at -80 degrees. These samples were later used for SCFA profiling on HPLC and were called "HPLC supernatant day 2". For Backlight staining 50 µl was taken out, where the rest of the medium with pellet was frozen at -20°C and would be later used for DNA quantification. These samples were called "qPCR day 2".

2.3 Viability testing of bacteria

Back light Bacterial staining is a method used to monitor the viability of bacterial populations. Cells that have damaged cytoplasmic membranes are considered dead and will stain red while cells with intact membranes are considered alive and will stain green (Berney et al., 2007). Back light staining was performed as a control of bacterial viability and the life stage of the different strains. The 50 µl of supernatant taken from the anaerobic chamber was within 1 hour mixed with 0,5 µl of back light staining colour (LIVE/DEAD Backlight TM, Bacterial Viability Kit. Invitrogen TM Molecular probes). All samples were kept dark for 15 minutes before microscope on a UV-microscope.

2.4 DNA extraction and purification

The cell and its DNA are well protected my membranes. Cell lysis is the first step in protein extraction to enable access to the DNA. Cell lysis for the disruption of cell membranes can be done by mechanical lysis such as bead beating and is a method commonly used as other methods are less effective (Mao et al., 2010).

The sample called "qPCR day 0" and the sample called "qPCR day 2" were thawed. After defrosting the samples, they were centrifuged (VWR, by Hitachi Koki Co, Ltd.) at 13 000 rpm for 10 minutes. The supernatant was carefully removed leaving a clean pellet. The pellet was resuspended in 50 µl TE buffer and 150 µl S.T.A.R buffer (Roche molecular systems) and

transferred to microtubes filled with glass beads. The samples were then put in the fastprep (96 TM) on 18 000 rpm for 40sec x 2. The supernatant obtained after the fastprep was transferred to new Eppendorf tubes before a manual DNA extraction was performed. DNA extraction was performed using Nucleic acid extraction KIT (from LGC group) instructions were followed by the manufacturer. For a detailed description see appendix C.

2.5 Quantification of bacteria with Quantitative polymerase chain reaction

The eluate obtained from DNA extraction was used as template DNA for qPCR targeting 16S rRNA V3-V4 region. qPCR is a rapid method for nucleic acid detection and for quantifying the product based on fluorescent detection. The master mix containing forward primer 341F, revers primer 806 R primer and 5xHOTFIRE EvaGreen qPCR super mix was made following protocol for qPCR preparations targeting 16s rRNA V3-V4 region given by the manufacturer. For detailed description see appendix D. EvaGreen qPCR super mix is binding to the double stranded DNA (dsDNA) and can in this way be used for detection of 16s rRNA V3-V4 region. Detailed description of mastermix is given in appendix D. 18 µl of master mix was added to the qPCR trays with 2 µl of DNA template of each bacteria strain. LightCycler 480 II from Roche was used with the following program; 95 C for 15 minutes, 95 C for 30 seconds, 55 C for 30 seconds and 72 C for 45 seconds with 40 cycles in total. Controls were added with E. *Coli* as positive control and Nuclease free water to the super mix as a negative control.

Primer name	Sequence 5’ – 3’	Target Region
Forward 341 F	5’-CCTACGGGRBGCASCAG-3’	V3-V4
Revers 806 R	5’-GGACTTACYVGGGTATCTAAT-3’	V3-V4

Table 1: The table represents the primers that were used for the DNA quantification in all samples that were performed on qPCR.

The data obtained from the qPCR was imported to Bio-Rad CFX maestro and measurements of qPCR were analysed.

2.6 High performance liquid chromatography Sample preparation

Liquid chromatography is useful to help one to separate a mix of substances. The identification depends on standards that are being used or published retention times.
The supernatant marked “HPLC supernatant day 2” was thawed and centrifuged at 13 000 rpm for 5 minutes. Supernatant was carefully transferred to new Eppendorf tubes. It was decided to use a dilution, with a factor five, of the samples after doing the pilot study. Samples that are not acidic by nature must be acidified prior to the analysis therefore 120 µl

of H_2SO_4 5 mM was added to 30 µl of samples, with a total volume of 150 µl. All samples diluted with H_2SO_4 were transferred to filter tubes with pores of 0,2 µM (VWR international) and centrifuged at 13 000 rpm for 5 minutes. 30 µl of the filter sterilized supernatant was then transferred to HPLC vials.

Standards were prepared out of following SCFA; acetic acid, propionic acid, iso-butyric acid, butyric acid, isovaleric acid, valeric acid, and 10% formic acid in a serial dilution with following concentrations; 25 mM stock, 10 mM stock, 5 mM stock, 2.5 mM stock and 1 mM stock. HPLC was used for the analysis of SCFA using a HPLC from REZEX ROA-Organic Acid H+ (Phenomenex, Torrance, California, USA) 300×7.8 mm ion exclusion column, isocratic elution with 0.6 mL/min 4 mM H_2SO_4 at 65 °C and UV detection at 210 nm in collaboration with biorefinery lab and with guidance from Leszek Michalack.

The results were imported to Chromeleon 7, where the chromatographic profile was analysed. The data set was further imported to Excel were average, SD and SEM was calculated. Excel was used to make boxplots with SEM and to make colour coded tables.

3.0 Results

3.1 Pilot study

A pilot study was conducted prior to the main project to establish growth conditions, length of incubation and SCFA profile measurements were tested. There was not observed any significant differences between incubation times of 48h and 96h after DNA quantification with qPCR and it was therefore decided to only continue with incubation time of 48 h. For SCFA profiling samples were measured undiluted and diluted with a factor of 10 and 5. The most accurate results of the SCFA profile was obtained from the factor 5 dilution and it was therefor decided to continue with a that factor for the main study.

All protocols for the main project were made according to the results obtained in the pilot study.

3.2 Viability testing of bacteria

After the second day of bacteria incubation in different carbon sources an aliquot amount was taken from the sample for Backlight staining as an extra control point to visually quantify the growth and liveability of the bacteria strains. There was observed good viability for all strains. In all cases there was observed more than 80 % green stained bacteria, in a few cases minor amounts of bacteria was stained orange and rarely some bacteria were

stained red. It was therefore concluded that the growth conditions for Bifidobacteria were sufficient for obtaining trustworthy results of SCFA production.

3.3 Quantification of bacteria with qPCR

The qPCR was performed on the samples called qPCR day 2. From the three parallels an average of CT values was calculated for the samples with P-Y medium with no added carbon source and compared with samples added different carbon sources. The difference between the CT values was calculated as the different carbon sources subtracting the PY-medium. If the difference was less than one, it was categorized as no growth, difference between one and two was categorized as little growth and a difference above two was categorized as growth. The results were implemented in a colour coded table, represented in figure 4.

Positive control with *Escherichia coli* was added with a CT value of 28,33, confirming that the qPCR run was reliable.

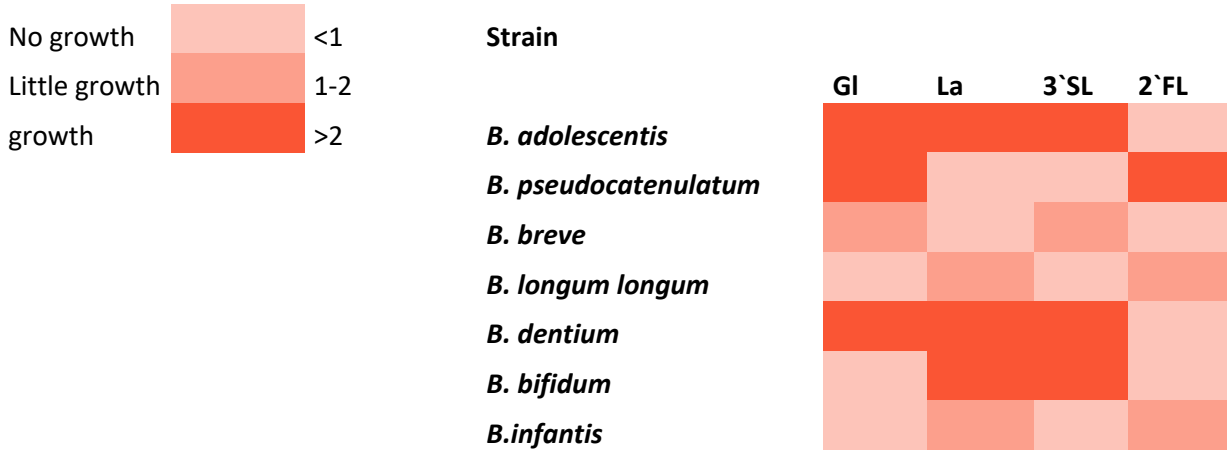

Figure 4: A colour coded representation of bacteria quantification, a comparison between samples. The CT values of all samples from the 3 parallels were collected and an average was calculated. From the three parallels an average of CT values was calculated for the samples with P-Y medium with no added carbon source and compared with samples added different carbon sources. No growth was categorized as differences below 1 in CT value. Little growth was categorized as differences of 1-2 and growth was categorized as anything over the difference of 2 in CT value.

B. adolesescentis was most prone to growth together with *B. dentium* on all carbon sources except 2'FL (*B. adolescentis* had CT values of 21,9 in glucose, 13,75 in lactose and 21,14 in 3'SL and *B. dentium* 23,39, 23,08 and 24,21 respectively). *B. bifidum* had growth on la and 3'SL (CT values of 22,89 in lactose and 21,64 in 3'SL) while *B. infantis* exhibited growth in

lactose and 2'FL (with CT values of 21,08 and 21,32 respectively). There was no registered growth in lactose for *B.breve*, but growth in glucose (CT of 18,35) and 3'SL (CT of 18,06). For raw data of CT values see appendix E.

3.4 High performance liquid chromatography

For the SCFA profiling HPLC was used. The results from the three parallels were calculated and the average was used to make a colour coded representation of the SCFA production for each acid. Values below 1 mM were not considered as significant and have therefor been categorized as no production. The results for all acids are represented in figure 5- 10. The standard curve used for the calculations of SCFA concentrations is represented in appendix F, for all raw data of each bacteria strain see appendixes G-M.

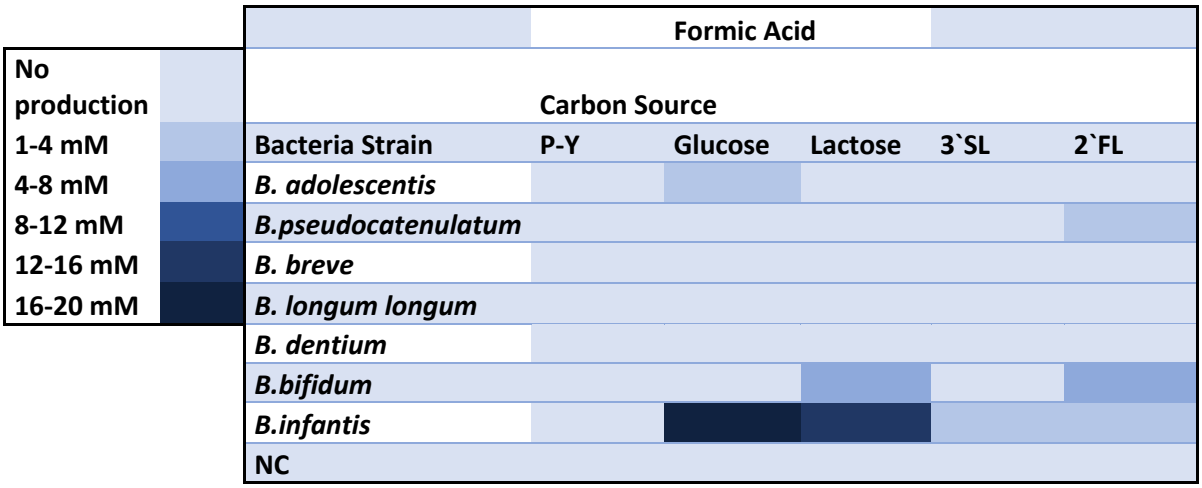

Figure 5. The figure represents the production of formic acid on each bacteria strain and in each carbon source. The figure represents lactic acid production from the different bacteria strains in different carbon mediums. The production of acetic acid is categorized into colour coded values were no production is all values below 1 mM, and rest is in the categorise of 1-10 mM.10-20mM and 20-30mM.

As represented in figure 5 most strains did not show any significant production of formic acid except of *B. bifidum* in lactose and 2'FL mediums (with values of 4,74 mM and 4,4 mM), and also quite high production was observed for *B. infantis* in glucose and lactose mediums (with average values of 19,7 mM and 12,9 mM respectively). Some minor production was also seen on 3'SL and 2'FL (1,7 mM and 2,7 mM) but there were big differences of SEM for the samples of glucose and lactose for *B. infantis* (SEM of 17,09 and 11,21 respectively).

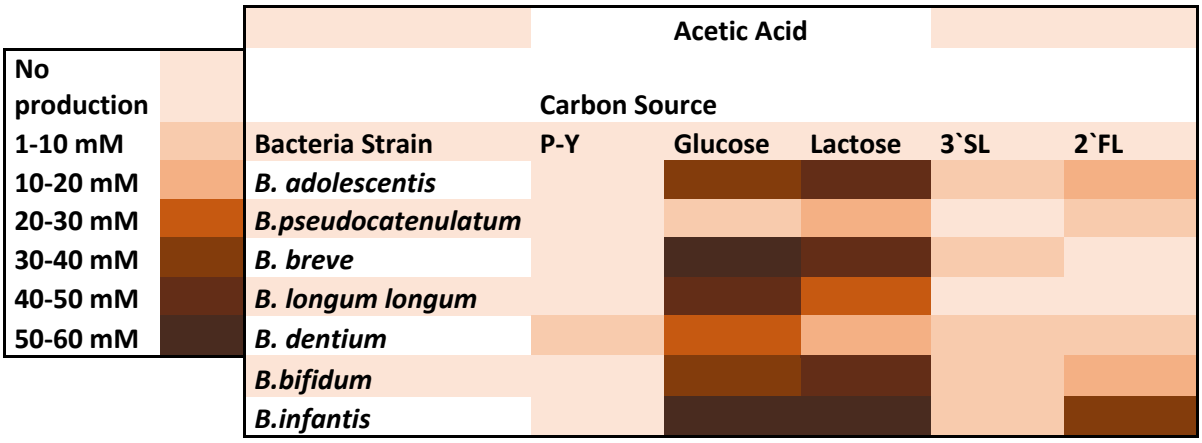

Figure 6. The figure represents the production of acetic acid on each bacteria strain and in each carbon source. The figure represents lactic acid production from the different bacteria strains in different carbon mediums. The production of acetic acid is categorized into colour coded values were no production is all values below 1 mM, and rest is in the categorise of 1-10 mM.10-20mM and 20-30mM.

For the production of acetic acid there was overall good production of acid for all strains on glucose, lactose and to lesser extent on 3'SL and 2'FL. *B. adolescentis* show generally high production in glucose and lactose and some production on 3'SL and 2'FL. *B. breve* has the highest production in glucose medium (57,8 mM), lactose (49,03 mM) and some in 3'SL (2,5 mM). *B. bifidum* also executed high production in glucose (30,2 mM) and lactose (41,1 mM) and relative high production in 3'SL (9,6 mM) and 2'FL (16,8 mM). The SEM in medium was glucose 9,91.

B. infantis had the absolute highest production of acetic acid of all strains with the highest production in glucose and lactose mediums (39,4 mM and 56,7 mM), but the glucose sample had high SEM values (SEM of 17,09 in glucose). Some minor production was observed in 3'SL medium (8,9 mM) and quite high values in 2'FL (34,4 mM), the sample of 2'FL had high values of SEM (15,6).

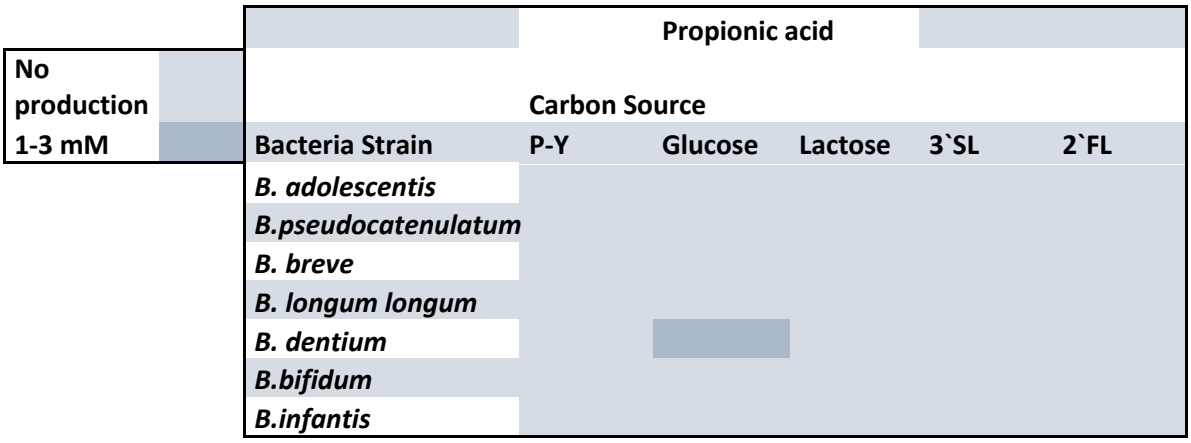

Figure 7. The figure is representing the production of propionic acid. The figure represents Lactic acid production from the different bacteria strains in different carbon mediums. The production of acetic acid is categorized into colour coded values were no growth is all values below 1 mM, and rest is in the categorise of 1-10 mM, 10-20mM and 20-30mM.

For the production of propionic acid there was only observed some production in glucose medium (2 mM) produced by *B. dentium*.

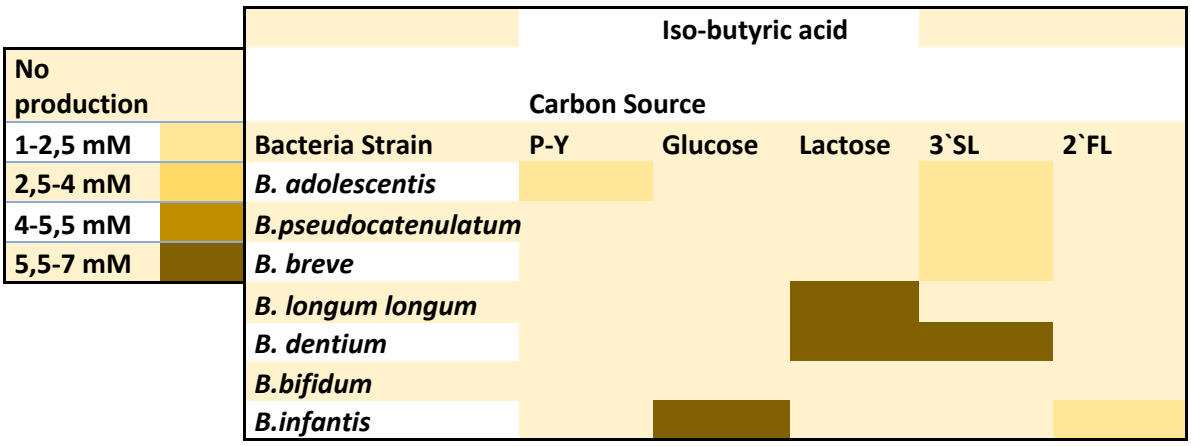

Figure 8. Iso-butyric production. The figure represents Lactic acid production from the different bacteria strains in different carbon mediums. The production of acetic acid is categorized into colour coded values were no growth is all values below 1 mM, and rest is in the categorise of 1-10 mM.10-20mM and 20-30mM.

Overall most bacteria strains did not produce iso-butyric acid, only some production was observed and mainly in 3'SL medium for all the strains. *B. longum* subsp. *longum*, *B. dentium* together with *B. infantis* showed highest production in glucose, lactose and 3'SL but all values were not higher that 5,5-7 mM. *B.infantis* was the only strain able to produce iso-butyric acid on 2'FL (1,06 mM).

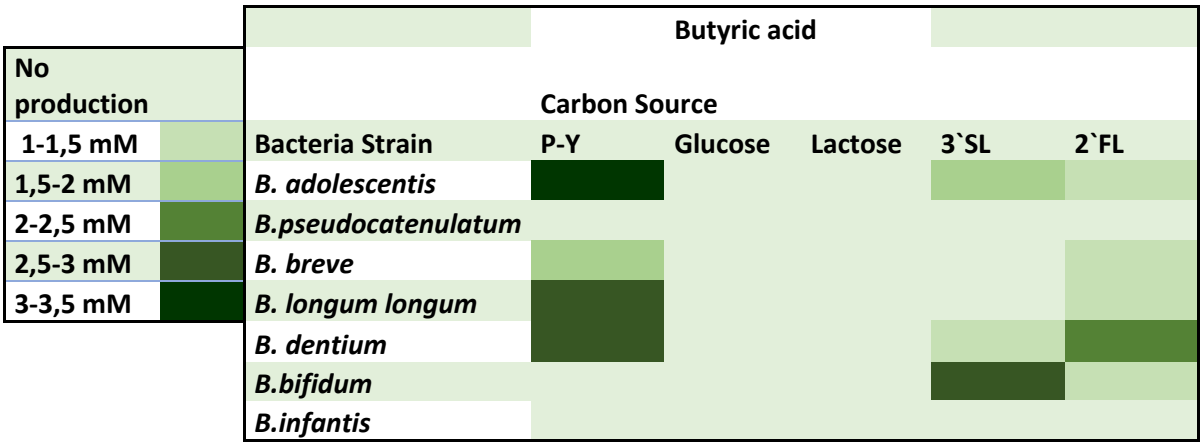

Figure 9. Butyric acid production. The figure represents Lactic acid production from the different bacteria strains in different carbon mediums. The production of acetic acid is categorized into colour coded values were no growth is all values below 1 mM, and rest is in the categorise of 1-10 mM.10-20mM and 20-30mM.

Overall the production of butyric acid was relatively low for all of the bacteria strains, with no values greater than 3,5 mM. There was not observed any production of butyric acid in either glucose or lactose for any of the strains, whereas most strains did show production in P-Y medium with no added carbon source, with the highest production for *B. adolescentis* (3,4 mM), *B. longum* subsp. *longum* (2,5 mM) and *B. dentium* (2,9 mM). *B. dentium* also showed some production in 3'SL (1,4 mM) and 2'FL (2,0 mM), B. bifidum in 3'SL (2,7 mM) and 2'FL (1,6 mM).

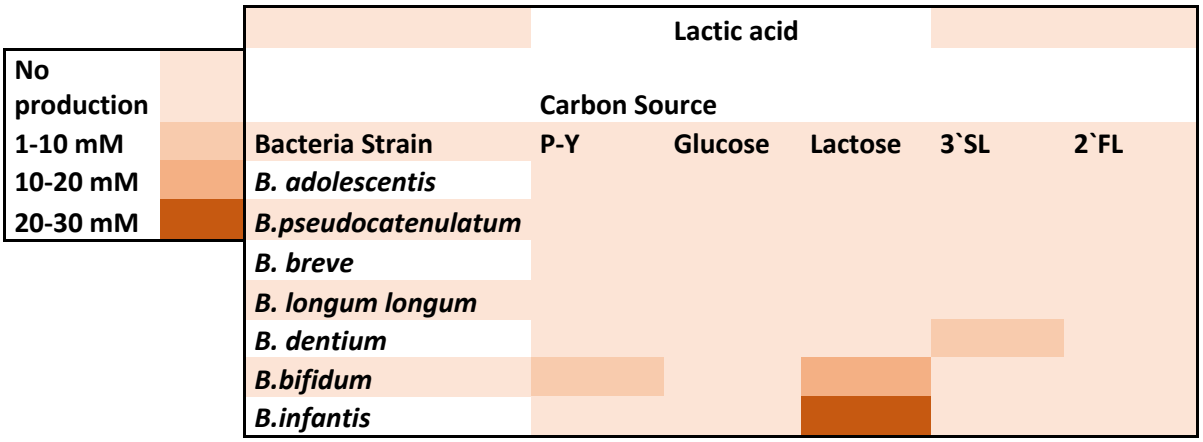

Figure 10. Lactic acid production. The figure represents Lactic acid production from the different bacteria strains in different carbon mediums. The production of acetic acid is categorized into colour coded values were no growth is all values below 1 mM, and rest is in the categorise of 1-10 mM.10-20mM and 20-30mM.

Generally, there was no production of lactic acid for most of the strains in the different carbon sources. *B. bifidum* did show production in P-Y medium with no added carbon source (4,4 mM) and quite high levels on lactose (17,91 mM). *B. infantis* had the highest values of all strains in

lactose medium (31,5 mM). *B. dentium* was the only strain showing any production of lactic acid on the selected oligosaccharides in 3'SL medium (3,19 mM).

Iso-Valeric acid and Valeric acid was not observed in any of the Bifidobacterial strains.

4.0 Discussion

4.1 main findings

The main findings of this thesis were *B. infantis* ability to produce significantly higher amounts of SCFA compared with the other strains, demonstrating *B. infantis* capability of utilizing HMO for growth and production of SCFA, results in accordance with Ruiz-Moyano et al and Pokusaeva et al.

The main metabolite of carbohydrate fermentation was acetic acid, supported by previous research performed by LeBlanc et al. The results did also confirm that the most common infant type lactic-utilizing and butyrate-producing bacterium Bifidobacteria (*B. dentium, B, bifidum* and *B. infantis*) are the main producers of lactic acid also supported by results of Bridgman et al. and Duncan et al (Bridgman et al., 2017, Duncan et al., 2009).

4.2 Quantification of bacteria growth

After cultivation of bacteria in different carbon sources the quantification of DNA with qPCR showed that the majority of bacteria had highest growth in either glucose or lactose mediums, followed by 3'SL medium. The strain had had most growth was *B. adolescentis* and *B. dentium* with good growth on all carbon sources except 2'FL medium. These results support research done by Zhou et al, also supported by Milani et al in the believe that *B. adolescentis* is able to partially utilize HMOs (Milani et al, 2017, Zhou et al)
B. bifidum did only grow in lactose medium and 3'SL, it was expected that this strain would also grow in 2'FL medium, as this is the most abundant type of HMO (Smilowitz et al., 2014).
B. infantis had growth in lactose and 2'FL medium. As lactose is the most abundant nutritional component in breast milk it is therefore to be expected that *B. bifidum* and *B. infantis* being one of the most common types of infant Bifidobacteria strains possesses this ability (Smilowitz et al., 2014, Ward et al., 2007).

4.3 SCFA profiling with the use of HPLC

The SCFA profile screening revealed acetic acid as the main product irrespective of both the carbon source and bacteria strain which is in accordance with LeBlanc et al, confirming acetate as one of the main metabolic produces of Bifidobacteria (LeBlanc et al., 2017). The production of acetic acid was followed by, in descending order, butyric acid, iso-butyric acid, formic acid, lactic acid and propionic acid. On the contrary valeric and iso-valeric acid production was not observed in any of the carbon sources by any bacterial strain.

Mediums evidently stimulating SCFA production were found to be glucose and lactose, corresponding with conclusions in another study (Ward et al., 2007). The results are also confirmed by qPCR DNA quantification results, which showed most growth in glucose and lactose solutions. The SCFA that was mainly produced on 2'FL was formic acid, acetic acid and butyric acid, while 3'SL had the highest production of acetic acid, iso-butyric acid and to some extent butyric acid suggesting that there was limitation in carbohydrate availability and the energy source is then consumed at a slower rate (O'Callaghan and van Sinderen, 2016, LeBlanc et al., 2017).

4.3.1 Production of Acetic acid

B. infantis was the strain that demonstrated the overall highest production of acetic acid where the potential role of acetic acid could be pathogenic protection in the infant gut (den Besten et al., 2013, Ríos-Covián et al., 2016) although high values of SEM were observed in glucose and 2'FL mediums. High SEM values may implicate deviating measurements in the three sample parallels.

Following *B. infantis* in acetate production was *B. breve* and *B. bifidum* which is conclusive with research by Thomson, Medina and Garrido and Matamoros et al, as these strains are the most prevalent in the infant gut (Thomson, Medina and Garrido, 2018, Matamoros et al., 2013) although a possible source of error was high values of SEM seen in the measurements in glucose medium of *B. bifidum* strain.

4.3.2 Production of butyric acid

Low production of butyric acid was observed in all of the infant type Bifidobacterial strains. The observed levels of butyrate production in 2'FL and 3'SL can indicate that Bifidobacteria can have low production of butyrate, supporting the idea that Bifidobacteria can produce butyrate (Wopereis et al 2014). Some claim that Bifidobacterial species do not produce

butyrate at all, but that it`s rather a product of *Bacteriocides* phylum and Clostridium (O'Callaghan and van Sinderen, 2016). Research performed by Pokusaeva et al confirmed *B. longum* and *B. bifidus* ability of butyrate production, while Pham et al suggests that a general low observed concentration of faecal butyrate in the infant gut can be due to the low levels of lactic-utilizing and butyrate-producing bacterium (LUBs) (Pham et al., 2016, Pokusaeva, Fitzgerald and van Sinderen, 2011).

In this experiment there was observed some butyric acid production in P-Y medium with no carbon source, this might be due to MRS mediums nutritional richness (and therefore not being highly selective). Marcobal et al performed test growth of lactic acid bacteria on MRS medium lacking any added sugar and results showed significant background growth making testing for HMO growth problematic, as it is impossible to distinguish moderate or even weak growth on HMO from background growth (Marcobal et al., 2010). It was therefore suggested to use a common medium for which one can manipulate the carbohydrate source when comparing carbohydrate consumption by many different species or strains (Marcobal et al., 2010). MRS medium is often added a common growth medium (such as nutritionally rich and complex yeast extract) due to the diverse nutritional requirements of intestinal microbes. It has been suggested to use a defined medium which can support good cell growth and be better suited for HMO consumption study by selected microbes (Marcobal et al., 2010).

It is hypothesised that the typical infant gut milieu may play a critical role in the establishment of LUBs, as research display poor establishment of LUBs in infants with eczema aged 26 weeks (Wopereis et al., 2018). The differences in occurrence of eczema may demonstrate the importance of LUBs guiding role in the process of transition from only liquid to the introduction of solid foods (which usually happens between 4-6 months of age). Even though butyrate is not the most abundant SCFA in infant's, colonization of low numbers early in life is thought to aid in the transition of microbes during the process of weaning. In the transition period the microbiota goes from lactate and acetate dominating milieu (from a Bifidobacterium dominating microbiota) to a more adult like butyrogenic milieu (to a microbiota rich in *Bacteriocides* spp and clostridium spp) (Milani et al, 2017, Wopereis et al., 2018). This hypothesis has been confirmed as there has been observed a correlation between specific microbial establishment and the onset and severity of eczema

in 6-month old infants and an inverse correlation with elevated levels of butyrate producing bacteria with an alleviation of eczema symptoms (Nylund et al., 2015).

4.3.3 Production of propionic acid

No production of propionic acid was observed in any of the strains. Studies performed by Pokusaeva et al together with LeBlanc et al demonstrated *B. longum* and *B. bifidum`s* ability of propionic acid production in MRS medium (Pokusaeva, Fitzgerald and van Sinderen, 2011, LeBlanc et al., 2017) therefore results obtained in this study did not confirm or correspond with their findings. The lack of propionic acid production could possibly be due to a poor MRS medium in our study and a repeat run with altered MRS medium should have been considered. Moreover, there is little information and scarce available literature on propionic acid production by Bifidobacteria. The lack of depth in research and literature becomes a constraint in further discussion of this subject.

4.3.4 Lactic acid production

B. bifidum exerted high levels of lactic acid production in lactose medium but not in any of the HMO derivatives. Genomic analysis performed by Sela et al. reported *B. bifidum* to be able of host derived glycan utilization (Sela et al., 2008) complementary studies have also demonstrated *B. bifidum's* ability to utilize LNB structure (LoCascio et al., 2007, Yu, Chen and Newburg, 2013) which was not observed in this experiment.

4.3.5 Utilization of HMO

Although there is consensus that Bifidobacteria species are able to utilize and grow on HMOs there is not a clear agreement to which of the strains possess this ability to either full or partial utilization or lack the ability all together. The body of literature is not consistent. In research performed by Pokusaeva et al *B. breve* together with *B. infantis* were two of the strains that exhibited the most effective HMO utilization. This ability is believed to be due to LNBs ability to support growth of these strains (Pokusaeva, Fitzgerald and van Sinderen, 2011, Xiao et al., 2009, LoCascio et al., 2007, Strum et al., 2012).

Contradicting findings are presented by Ruiz-Moyano et al supporting the belief that *B. breve* has only the ability of partial HMO utilization (Ruiz-Moyano et al., 2013). Xiao et al reported in their study *B. breves* ability to grow on HMO monomer constituents suggesting a possible cross-feeding capacity in the GIT via liberated monosaccharides (Xiao et al., 2009, Asakuma et al., 2011). Our findings showed minor acetic acid production by *B. breve* in 3'SL supporting

the believe that *B. breve* is able of utilising HMO structures. It is noteworthy to mention that specifically *B. breve* has been related to lower risk of developing eczema and 2'FL has been related to the abundance of *B. breve* in the GIT (Ismail et al., 2016) even though there are also other genetic, epigenetic and environmental factors that affect the development of the disease (Milani et al, 2017).

B. dentium has previously been reported to be incapable of LNB utilization (Xiao et al., 2009) although in this experiment *B. dentium* was observed to produce acetic acid on all carbon sources and therefore the results are not supporting the findings of Xiao et al.

There are conflicting opinions weather *B. adolescentis* has the ability of HMO utilization or not. Zikovic et al and Pokusaeva et al claim through their research that *B. adolescentis* have no ability to utilize HMOs, neither in the form of LNT or liberated HMO monomers (Zivkovic et al., 2013, Pokusaeva, Fitzgerald and van Sinderen, 2011, Sela and Mills, 2010). While Milani et al presents results showing *B. adolescentis* ability of partial utilization, also confirmed by findings of Zhou et al (Milani et al, 2017, Zhou et al). Results obtained in this study are supporting these findings, as there was observed detectable amounts of acetic, iso-butyric and butyric acid in both 2'FL and 3'SL.

The observed and reported variations in SCFA production and HMO utilization of Bifidobacterial species suggest that there is still uncertainty about the metabolic utilization abilities of each strain and even within the strains there are observed great differences (Ruiz-Moyano et al., 2013). Research is also documenting that HMO utilization is not a property of all Bifidobacteria and that the full utilization capability is conserved to selected bifido strains (LoCascio et al., 2007). This deviating capability of HMO utilization by typical infant strains is suggested to be a confirmation of the mutualistic relationship and mutual advantage that breast milk and Bifidobacteria has co-evolved. In other words, the diversity of HMOs in breast milk is not only present to provide direct nutrition to the infant but is has also evolved as a specific substrate for aiding and supporting growth of beneficial Bifidobacteria that can aid and protect in the development of the new-born (LoCascio et al., 2007).

4.4 Methodological considerations

The body of literature is as presented, inconclusive in which type of Bifidobacteria strain has the ability of HMO utilization and also to which extent the strain can utilize HMO. There are

reported variations, although the same materials and conditions apply, to such a magnitude, that it becomes an issue to get a clear reference area of the concentrations. SCFA levels from the same materials in different studies, making it difficult to obtain a distinct area of concentrations for reference. This may be caused both by the volatile nature of SCFA and the SCFA utilization patterns of the body. The variating SCFA values were reflected by the deviating SEM values obtained in each parallel. To decrease both SEM values and the possibility of misinterpretation of data more parallels should be added.

Another limitation of this study is the HPLC interpretation methods. It can be difficult to recognize and differentiate between SCFA peaks from other peaks in the analytical program, thus individual interpretations may result in deviating results of reported SCFA concentration levels. To mitigate misinterpretation of results additional parallels should`ve been considered and other mediums should have been considered.

Due to the higher production of formic acid compared with butyric acid it could have been considered to add supplementary samples with higher concentrations of oligosaccharide concentrations to evaluate if that could have altered the SCFA production.

4.5 Concluding remarks

It has been clearly demonstrated that *B.infantis* possesses the best ability to utilize and grow from HMO, which may reflect their abundance in the breastfed infant gut.

In conclusion the results demonstrated differences in HMO utilization and SCFA production among the different strains. It was clearly demonstrated that *B. Infantis* has the best utilization and growth abilities on HMOs which may reflect their abundance in the infant gut. The main metabolite of carbohydrate fermentation performed by Bifidobacteria strain was acetic acid and the results also confirmed that the most common infant type Bifidobacteria strains are the main producers of lactic acid. Our findings did not support that Bifidobacteria produce propionic acid.

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Appendixes

Appendix A: Making of the Mrs+L-Cysteine medium

De Man Rogosa and Sharpe (MRS) from Sigma Aldrich with L- cysteine was used for bifidobacterial growth in following concentrations:

Medium	Amount (grams)
MRS medium powder	26
L-Cysteine	0,25
Agar powder	7,5
Milli Q	0,5 Liters*

Add the dry powders before adding MilliQ water, the total amount should be 0,5 L with all ingrediencies added. Mix the mediums in a 0,5 L glass bottle and mix well before autoclaving. Poor on plates after autoclaving and allow to set. Store at 4 °C.

Appendix B: Preparations for mediums and solutions

Preparation of Salt solution

For optimum growth conditions for Bifidobacteria

Medium	Amount (grams)
CaCl2	0,2
MgSO4	0,2
K2HPO4	1,0
KH2PO4	1,0
NaHCO3	10,0
NaCl	2,0
Final Volume	1 L

Preparing of the Peptone-Yeast extract

Medium	Volume 100 ml/g	Volume 500 ml/g
Peptone	0,5 g	2,5 g
Tryptone	0,5 g	2,5 g

Yeast extract	1,0 g	5,0 g
Salt solution	4 ml	20 ml
Milli Q	100 ml*	430 ml*

*Total volume should be either 100 ml or 500 ml, take this into account when measuring other ingredients.

Insert all dry ingrediencies in a blue cap glass bottle and insert magnet. Add MilliQ water to the desired total volume. Mix well and autoclave before use.

Preparing of 10% Glucose and Lactose solution

To make a stock solution with a 10 % final concentration weigh 1 g of glucose or lactose powder, add MilliQ water with a total weight of 10 g. Mix with magnet.

Filter sterilize before use. Do not autoclave as heating may alter the sugars properties.

Making the 10% Oligosaccharide solution

Medium	Volume (g)
Oligosaccharide 2FL	0,05 g
3 SL	0,05 g
MilliQ water	0,45 µl

To make stock solutions with a 10% final concentration weigh 0,05 g of the selected oligosaccharide, add MilliQ water till you reach a total weight of 0,5 g. You will then have a stock solution of a total volume of 0,5 ml with 10% oligosaccharide concentration. Filter sterilize before use.

Appendix C :Protocol for manual DNA extraction

After having the samples in fastprep for cell lysis the samples are ready for DNA extraction. To make sure the samples are homogenous centrifuge at 13 000 rmp for 5 minutes. Carefully take out 50 µl of the supernatant to new Eppendorf tubes. Discard the pellet.

Add 50 µl Lysis buffer BLm. Add 5 µl proteinase K to all the samples, mix by pipetting up and down carefully. Incubate at 55 °C for 10 minutes and let it cool down to room temperature.

Add 50 µl of 96% ethanol. Make sure all Mag particle suspension is properly mixed and add 16 µl of Mag particle suspension to all samples, wait 2 minutes allowing the particles to bind

to DNA fragments. Take the magnet in contact with the samples and remove the supernatant. Remove the magnet and add 170 µl of Wash Buffer BLm1 and resuspend the pellet. Incubate at room temperature for 10 minutes and mix the samples in between. Bring the magnet in contact with the samples and wait 1 minute. Remove and discard the supernatant. Repeat the steps from adding wash buffer times 2 but use Wash buffer 2 BLm for these steps.

Allow the pellet to dry at 55°C for 6 minutes leaving the tubes open allowing all liquid to evaporate.

Add 63 µl of Eluation buffer BLm and resuspend the pellet. Incubate at 55°C for 10 minutes and mix the samples in between. Bring the magnet in contact with the samples and wait for 3 minutes. Remove the eluate and transfer it to new test tubes. To avoid particles to mix with the eluate only transfer 50µl. Store the eluate at -20°C.

Appendix D: Protocol for qPCR preparations targeting 16s rRNA v3-v4 region:

Mediums	Initial concentration	Final concentration	Volume 1 rxn (µl)
5XHOTFIREPOL EVAGREENqPCR SUPERMIX	5 x	1x	4
Forward primer 341F	10 µM	0,2 µM	0,4
Revers primer 806R	10 µM	0,2 µM	0,4
Nuclease free Water	NA	NA	13,2
Template DNA		0,1-10 ng	2 µl
Final Volume			20 µl

Make the master mix by adding 5X HOTFIRE EVAGREEN qPCR SUPERMIX with forward primer 341 F and revers Primer 806R, then add the Nuclease Free water in a tube. Mix well.

Add 18 µl of the master mix to qPCR tray wells, then add 2µl of the DNA template to each well. Total volume is can maximum be 20 µl.

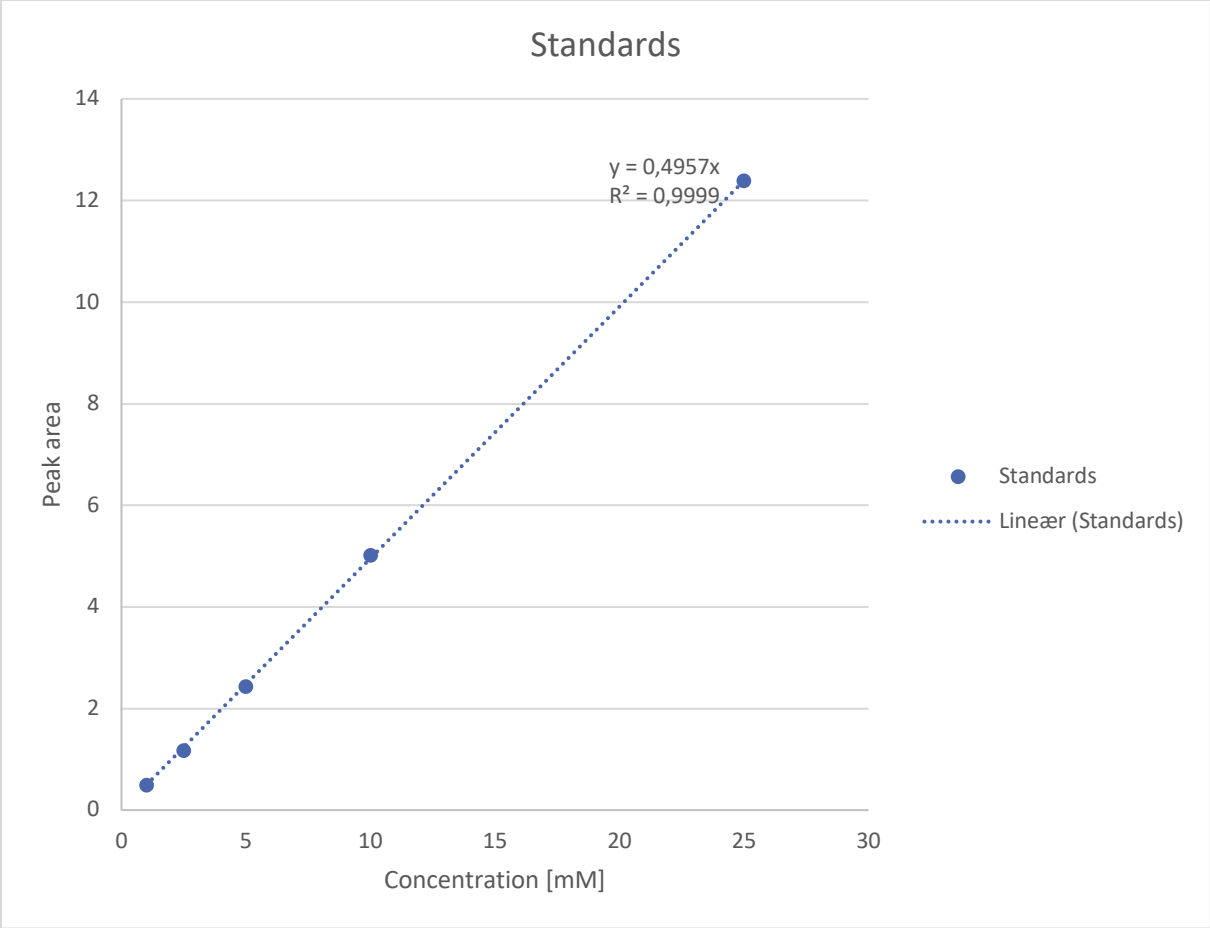
Add a known bacteria to a positive control well and 2 µl of nuclease free water to the negative control.

Appendix E: CT values for qpcr results

Strain	P -Y value day 2	Carbon source Glucose day 2
B.adolescentis	**24,08**	**21,9**
B.pseducatenulatum	**21,62**	**19,59**
B.breve	**19,9**	**18,35**
B. longum longum	**21,85**	**22,54**
B. dentium	**25,91**	**23,39**
B.Bifidum	**24,85**	**24,45**
B.infantis	**22,26**	**23,0**
Positive controll	**28,33**	
Strain	**P -Y value day 2**	**Carbon source lactose**
B.adolescentis	**24,08**	**13,75**
B.pseducatenulatum	**21,62**	**28,08**
B.breve	**19,9**	**19,86**
B. longum longum	**21,85**	**20,14**
B. dentium	**25,91**	**23,08**
B.Bifidum	**24,85**	**22,89**
B. infantis	**22,26**	**21,18**
Strain	**P -Y value day 2**	**Carbon source 3`SL**
B.adolescentis	**24,08**	**21,14**

B.pseducatenulatum	**21,62**	**23,91**
B.breve	**19,9**	**18,6**
B. longum longum	**21,85**	**22,56**
B. dentium	**25,91**	**24,21**
B.Bifidum	**24,85**	**21,64**
B. infantis	**22,26**	**23,06**
Strain	**P -Y value day 2**	**Carbon source 2`FL**
B.adolescentis	**24,08**	**26,0**
B.pseducatenulatum	**21,62**	**19,87**
B.breve	**19,9**	**21,77**
B. longum longum	**21,85**	**22,83**
B. dentium	**25,91**	**28,14**
B.Bifidum	**24,85**	**28,79**
B. infantis	**22,26**	**21,32**

Appendix F: Standard Curve



Appendix G : SCFA values for B.adolescentis

						Ligning standardkurva 0,49570			Oppregning for fortynning 5,00000						
strain	Carbon source	Type of SCFA	Replicate 1	Replicate 2	Replicate 3	Concentration rep 1 mM	Concentration rep 2 mM	Concentration rep 3 mM	Concentration rep 1 mM	Concentration rep 2 mM	Concentration rep 3 mM		Average	SD	SEM
DMS 20083		Formic acid	mAU*min	mAU*min	mAU*min										
	Py		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Glucose		0,60340	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Lactose		0,28510	0,00000	0,00000	0,47367	0,00000	0,00000	2,36837	0,00000	0,00000		0,78946	1,11646	0,644588115
	3'SL		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	2'FL		0,00000	0,42800	0,00000	2,91648	1,05669	0,00000	14,58241	5,28344	0,00000		6,62195	6,02801	3,480273621
		Acetic acid													
	Py		0,00040	0,00000	0,00000	0,00081	0,00000	0,00000	0,00403	0,00000	0,00000		0,00134	0,00190	0,001098106
	Glucose		5,43780	0,82610	4,53190	10,96994	1,66653	9,14242	54,84971	8,33266	45,71212		36,29816	20,12338	11,61824413
	Lactose		5,17390	3,82080	5,84680	10,43756	7,70789	11,79524	52,18782	38,53944	58,97620		49,90115	8,49850	4,906614101
	3'SL		0,00000	0,00000	0,74960	0,00000	0,00000	1,51220	0,00000	0,00000	7,56102		2,52034	3,56430	2,057850303
	2'FL		0,00000	4,46210	0,00000	9,00161	0,00000	0,00000	45,00807	0,00000	0,00000		15,00269	21,21701	12,24964493
		Propionic acid													
	Py		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Glucose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Lactose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	3'SL		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	2'FL		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
		Iso-butyric acid													
	Py		0,00000	0,00000	1,79890	0,00000	0,00000	3,62901	0,00000	0,00000	8,99450		2,99817	4,24005	2,447992832
	Glucose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Lactose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	3'SL		0,00000	0,37070	0,88680	0,00000	0,74783	1,78899	0,00000	1,85350	4,43400		2,09583	1,81827	1,049775964
	2'FL		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
		Butyric acid													
	Py		0,28180	0,00000	1,79890	0,56849	0,00000	3,62901	1,40900	0,00000	8,99450		3,46783	3,95005	2,280562971
	Glucose		0,15050	0,16760	0,00000	0,30361	0,33811	0,00000	0,75250	0,83800	0,00000		0,53017	0,37651	0,217375805
	Lactose		0,21860	0,20320	0,00000	0,44099	0,40993	0,00000	1,09300	1,01600	0,00000		0,70300	0,49809	0,287571827
	3'SL		0,18370	0,19310	0,88680	0,37059	0,38955	1,78899	0,91850	0,96550	4,43400		2,10600	1,64626	0,950466581
	2'FL		0,61780	0,00000	0,00000	1,24632	0,00000	0,00000	3,08900	0,00000	0,00000		1,02967	1,45617	0,840719313
		Iso-valeric acid													
	Py		0,13600	0,00000	0,00000	0,27486	0,00000	0,00000	0,68000	0,00000	0,00000		0,22667	0,32056	0,185072558
	Glucose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Lactose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	3'SL		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	2'FL		0,16910	0,00000	0,00000	0,34113	0,00000	0,00000	0,84550	0,00000	0,00000		0,28183	0,39857	0,230115953
		valeric acid													
	Py		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Glucose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Lactose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	3'SL		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	2'FL		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
		Lactic acid													
	Py		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Glucose		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0
	Lactose		0,00140	0,00000	0,00000	0,00282	0,00000	0,00000	0,00700	0,00000	0,00000		0,00233	0,00330	0,001905159
	3'SL		0,00000	0,00000	0,19710	0,00000	0,00000	0,39762	0,00000	0,00000	0,98550		0,32850	0,46457	0,268219127
	2'FL		0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000	0,00000		0,00000	0,00000	0

Appendix H: SCFA values for B.pseudocatenulatum

Standard curve 0,4957

Compensation for dilution 5

strain	Carbon so	Type of SC	Replicate 1	Replicate 2	Replicate 3	Concentra	Concentra	Concentra	Concentra	Concentration rep 3 m	Average	SD	SEM
DMS 20438		Formic aci	mAU*min	mAU*min	mAU*min								
	Py		0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0
	Lactose		0,2348	0	0,473674	0	0	1,174	0	0,391333	0,553429	0,319522	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0
	2'FL		1,4457	0,5238	0	2,916482	1,056688	0	7,2285	2,619	0	3,2825	2,368085	1,725172
		Acetic acid											
	Py		0	0	0	0	0	0	0	0	0	0	0
	Glucose		0,8261	0	1,3745	1,666532	0	2,772846	4,1305	0	6,8725	3,667667	2,824709	1,630847
	Lactose		3,8208	0	2,4827	7,707888	0	5,008473	19,104	0	12,4135	10,50583	7,914969	4,569709
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		4,4621	1,0243	0	3,001614	2,066371	0	22,3105	5,1215	0	9,144	9,542012	5,509083
		Propionic acid											
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Iso-butyric acid											
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0,399	0	0	0,804922	0	0	1,995	0	0,665	0,940452	0,54297
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	1,367359	0	0	3,389	0	0	1,129667	1,59759	0,922369
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Butyric acid											
	Py		0,1779	0	0,2651	0,358886	0	0,534799	0,8895	0	1,3255	0,738333	0,551589	0,31846
	Glucose		0	0	0,2062	0	0	0,415977	0	0	1,031	0,343667	0,486018	0,280603
	Lactose		0,1493	0	0	0,30119	0	0	0,7465	0	0	0,248833	0,351903	0,203172
	3'SL		0	0,235	0	0	0,474077	0	0	1,175	0	0,391667	0,5539	0,319794
	2'FL		0,2266	0	0	0,457131	0	0	1,133	0	0	0,377667	0,534101	0,308364
		iso-valeric acid											
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		valeric acid											
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Lactic acid											
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0

Appendix I: SCFA values for B.breve

					Standard Curve					Compensation for dilution				
					0,4957					5				
strain	Carbon so	Type of SC	Replicate 1	Replicate 2	Replicate 3	Concentra	Concentra	Concentra	Concentra	Concentra	Concentration rep 3 m	Average	SD	SEM
DMS 20213		Formic aci	mAU*min	mAU*min	mAU*min									
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		1,6504	0,0001	2,0072	3,329433	0,000202	4,049223	16,64717	0,001009	20,24612	12,2981	8,818613	5,091428
	Lactose		0	0	0,4467	0	0	0,90115	0	0	4,505749	1,501916	2,124031	1,226311
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Acetic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		4,5319	6,2542	6,4157	9,142425	12,61691	12,34271	45,71212	63,08453	64,71354	57,83673	8,539145	4,964719
	Lactose		5,8463	2,9992	5,7388	11,79524	6,050434	11,57716	58,9762	30,25217	57,88582	43,03806	13,29109	7,673613
	3'SL		0,7496	0	0	1,512205	0	0	7,561025	0	0	2,520342	3,564301	2,057855
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Propionic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Iso-butyric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0,3373	0,2136	0	0,680452	0,430906	0	3,402259	2,154529	0	1,852263	1,405315	0,811359
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Butyric acid												
	Py		0,1494	0,309	0	0,301392	0,623361	0	1,50696	3,116805	0	1,541255	1,272661	0,734771
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0,1918	0,1424	0	0,386928	0,287271	0	1,934638	1,436353	0	1,123664	0,820178	0,47353
		iso-valeric acid												
	Py		0,1835	0	0	0,370184	0	0	1,850918	0	0	0,616973	0,872531	0,503756
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Lactic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0

Appendix J: SCFA values for B.longum subsp

						Standard Curve 0,4957			Compensation for dilution 5					
Strain	Carbon so	Type of SC	Replicate 1	Replicate 2	Replicate 3	Concentra	Concentra	Concentra	Concentra	Concentra	Concentration rep 3 m	Average	SD	SEM
DMS 20219		Formic aci	mAU*min	mAU*min	mAU*min									
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0,4188	0	0	0,844866	0	0	4,224329	0	1,40811	1,991368	1,149717
	Lactose		0,9918	0	0	2,000807	0	0	10,00403	0	0	3,334678	4,715947	2,722753
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		1,1303	0	0	2,28021	0	0	11,40105	0	0	3,80035	5,374506	3,102973
		Acetic acid												
	Py		0	0,5748	0	0	1,159572	0	0	5,797862	0	1,932621	2,733138	1,577978
	Glucose		5,8379	2,2639	3,8065	11,77708	4,567077	7,67904	58,88541	22,83538	38,3952	40,03867	14,76317	8,523522
	Lactose		3,7339	0	4,4127	7,53258	0	8,901957	37,6629	0	44,50978	27,3909	19,56895	11,29814
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Propionic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Iso-butyric acid												
	Py		0,9688	0	0	1,954408	0	0	9,77204	0	0	3,257347	4,606584	2,659612
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	1,8316	0	0	3,694977	0	0	18,47488	0	6,158295	8,709144	5,028227
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Butyric acid												
	Py		0,2487	0,235	0,2665	0,501715	0,474077	0,537624	2,508574	2,370385	2,688118	2,522359	0,130079	0,075101
	Glucose		0,0053	0	0	0,010692	0	0	0,05346	0	0	0,01782	0,025201	0,01455
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0,2077	0	0	0,419003	0	0	2,095017	0	0,698339	0,987601	0,570191
	2'FL		0	0,2009	0,2145	0	0,405285	0,432721	0	2,026427	2,163607	1,396678	0,989187	0,571107
		iso-valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0		0	0
		valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Lactic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0

Appendix K: SCFA values for B.dentium

						Standard Curve 0,4957			Compensation for dilution 5					
Strain	Carbon so	Type of SC	Replicate 1	Replicate 2	Replicate 3	Concentra	Concentra	Concentra	Concentra	Concentra	Concentration rep 3 m	Average	SD	SEM
DMS 20438		Formic aci	mAU*min	mAU*min	mAU*min									
	Py		2,5955	0	0	1,286589	0	0	6,432947	0	0	2,144316	3,03252	1,750826
	Glucose		1,1329	0	0	0,561579	0	0	2,807893	0	0	0,935964	1,323653	0,764212
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		1,5102	1,325	0	0,748606	0,656803	0	3,743031	3,284013	0	2,342348	1,666857	0,96236
	2'FL		1,9233	0	0	0,95338	0	0	4,766899	0	0	1,588966	2,247138	1,297386
		Acetic acid												
	Py		2,8858	0	1,1379	1,430491	0	0,564057	7,152455	0	2,820285	3,324247	2,941642	1,698358
	Glucose		5,5489	1,0667	1,1622	2,75059	0,528763	0,576103	13,75295	2,643816	2,880513	6,425759	5,182006	2,991833
	Lactose		4,2244	0	1,901	2,094035	0	0,942326	10,47018	0	4,711629	5,060601	4,281548	2,471953
	3'SL		1,6821	1,1691	0,9425	0,833817	0,579523	0,467197	4,169085	2,897614	2,335986	3,134228	0,766834	0,442732
	2'FL		2,0913	0	0	1,036657	0	0	5,183287	0	0	1,727762	2,443425	1,410712
		Propionic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0,6123	0	0	0,303517	0	0	1,517586	0	0	0,505862	0,715397	0,413034
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Iso-butyric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0,1099	1,8288	0	0,054477	0,906536	0	0,272387	4,532681	0	1,601689	2,075505	1,198293
	3'SL		0,8067	0,9604	0	0,399881	0,47607	0	1,999406	2,380351	0	1,459919	1,043968	0,602735
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Butyric acid				0	0	0	0	0	0	0	0	0
	Py		0,4182	0,1815	0,2705	0,207302	0,08997	0,134087	1,036509	0,449848	0,670434	0,71893	0,241946	0,139687
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0,1566	0	0	0,077627	0	0	0,388133	0,129378	0,182968	0,105636
	3'SL		0,1158	0,151	0,1512	0,057402	0,074851	0,07495	0,28701	0,374254	0,374749	0,345338	0,041244	0,023812
	2'FL		0,1811	0,264	0,1558	0,089771	0,130865	0,07723	0,448856	0,654324	0,38615	0,496444	0,114536	0,066127
		iso-valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Lactic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0,9496	0	0	0,470717	0	0	2,353584	0	0	0,784528	1,10949	0,640564
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0

Appendix L: SCFA values for B.bifidum

						Standard Curve 0,4957		Oppregning for fortynning 5						
strain	Carbon so	Type of SC	Replicate 1	Replicate 2	Replicate 3	Concentra	Concentra	Concentra	Concentra	Concentra	Concentration rep 3 m	Average	SD	SEM
DMS 20456		Formic aci	mAU*min	mAU*min	mAU*min									
	Py		0,6082	0,4608	0	1,226952	0,929595	0	6,134759	4,647973	0	3,594244	2,61299	1,50861
	Glucose		1,1581	0,8478	0	2,336292	1,710309	0	11,68146	8,551543	0	6,744335	4,937181	2,850483
	Lactose		1,4114	0	0	2,847287	0	0	14,23643	0	0	4,745478	6,711119	3,874666
	3'SL		2,0093	0	0	4,05346	0	0	20,2673	0	0	6,755766	9,554096	5,51606
	2'FL		1,3201	0	0	2,663103	0	0	13,31551	0	0	4,438504	6,276993	3,624024
		Acetic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		3,9131	0,8361	4,2422	7,894089	1,686706	8,557999	39,47045	8,433528	42,78999	30,23132	15,47283	8,933243
	Lactose		5,5184	4,5483	2,1702	11,13254	9,175509	4,378051	55,6627	45,87755	21,89026	41,1435	14,18809	8,191498
	3'SL		2,3306	0,5233	0	4,701634	1,055679	0	23,50817	5,278394	0	9,595521	10,07097	5,814478
	2'FL		5,0062	0	0	10,09925	0	0	50,49627	0	0	16,83209	23,80417	13,74334
		Propionic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Iso-butyric acid												
	Py		0	0,2713	0	0	0,547307	0	0	2,736534	0	0,912178	1,290015	0,74479
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Butyric acid												
	Py		0	0	0,2417	0	0	0,487593	0	0	2,437967	0,812656	1,149268	0,66353
	Glucose		0	0,2182	0	0	0,440186	0	0	2,200928	0	0,733643	1,037527	0,599017
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0,4912	0,1581	0,1645	0,990922	0,318943	0,331854	4,95461	1,594715	1,65927	2,736198	1,568875	0,905791
	2'FL		0	0,278	0,212	0	0,560823	0,427678	0	2,804115	2,13839	1,647502	1,196243	0,690651
		iso-valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Lactic acid												
	Py		0	1,3171	0	0	2,657051	0	0	13,28525	0	4,428418	6,262728	3,615788
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		5,33	0	0	10,75247	0	0	53,76236	0	0	17,92079	25,34382	14,63226
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0,0005	0	0	0,001009	0	0	0,005043	0	0,001681	0,002377	0,001373

Appendix M: SCFA values for B.infantis

						Ligning standardkurva 0,4957			Oppregning for fortynning 5					
strain	Carbon so	Type of SC	Replicate 1	Replicate 2	Replicate 3	Concentra	Concentra	Concentra	Concentra	Concentra	Concentration rep 3 m	Average		
DMS 20088		Formic aci	mAU*min	mAU*min	mAU*min									
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		2,1415	5,8705	0	4,320153	11,84285	0	21,60077	59,21424	0	26,93834	24,46697	14,12601
	Lactose		0,7091	3,8524	0	1,430502	7,771636	0	7,152512	38,85818	0	15,3369	16,88644	9,749389
	3'SL		0,5069	0	0	1,022594	0	0	5,112972	0	0	1,704324	2,410278	1,391575
	2'FL		0	0	0,8243	0	0	1,662901	0	0	8,314505	2,771502	3,919495	2,262922
		Acetic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		5,8531	5,8705	0	11,80775	11,84285	0	59,03873	59,21424	0	39,41766	27,87259	16,09224
	Lactose		6,6734	3,8524	6,2804	13,46258	7,771636	12,66976	67,31289	38,85818	63,3488	56,50662	12,58383	7,265278
	3'SL		0,8025	0	1,8452	1,618923	0	3,722413	8,094614	0	18,61206	8,902226	7,619773	4,399278
	2'FL		4,8723	0	5,3633	9,829131	0	10,81965	49,14565	0	54,09824	34,41463	24,41867	14,09813
		Propionic acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Iso-butyric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0,2558	1,7671	0	0,516038	3,564858	0	2,58019	17,82429	6,801493	7,86515	4,540946
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0,3158	0	0	0,637079	0	0	3,185394	0	0	1,061798	1,501609	0,866955
		Butyric acid												
	Py		0	0	0,2673	0	0	0,539237	0	0	2,696187	0,898729	1,270995	0,733809
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		iso-valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		valeric acid												
	Py		0	0	0	0	0	0	0	0	0	0	0	0
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		0	0	0	0	0	0	0	0	0	0	0	0
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0
		Lactic acid												
	Py		0,2705	0	0	0,545693	0	0	2,728465	0	0	0,909488	1,286211	0,742594
	Glucose		0	0	0	0	0	0	0	0	0	0	0	0
	Lactose		9,3692	0	0	18,90095	0	0	94,50474	0	0	31,50158	44,54996	25,72093
	3'SL		0	0	0	0	0	0	0	0	0	0	0	0
	2'FL		0	0	0	0	0	0	0	0	0	0	0	0

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