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University of Turku

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The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin Originality Check service.

ISBN 978-951-29-8204-2 (PRINT)
ISBN 978-951-29-8205-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy, Turku, Finland 2020

Ei sitä ääneen sanota,
rakkaus näkyy teoissa
(Eerika Salli,
Metsämiehen laulu)

UNIVERSITY OF TURKU
Faculty of Medicine
Institute of Dentistry
Periodontology
KRISTA SALLI: Modulation of oral and gut microbiota with xylitol and 2´-fucosyllactose: *in vitro* studies
Doctoral Dissertation, 139 pp.
Finnish Doctoral Program in Oral Sciences – FINDOS Turku
November 2020

ABSTRACT

The gastrointestinal tract starts at the oral cavity and ends at the colon, and both habitats are heavily colonised with microbes. The microbial communities are modulated by food ingredients such as sugar, xylitol and human milk oligosaccharides (HMOs). This thesis aimed to evaluate: 1) the effects of xylitol and an HMO, 2´-fucosyllactose (2´-FL), on the growth, adhesion and biofilm formation of caries associated bacterium *Streptococcus mutans;* and 2) the effects of 2´-FL on simulated infant microbiota and metabolite compositions. We utilised *in vitro* model systems to mimic the oral cavity and colon.

The biofilm formation of mutans streptococci was decreased in the presence of xylitol and xylitol mints and increased in the presence of sucrose and sucrose mints. Planktonic *S. mutans* grew well on galacto-oligosaccharides (GOS), whereas 2´-FL was not utilised by *S. mutans* as a carbon source, and xylitol inhibited *S. mutans* growth. The adhesion experiments showed no consistent inhibition patterns for 2´-FL or GOS, and 1% xylitol did not inhibit the adhesion of the *S. mutans* strains. 2´-FL, GOS and lactose all promoted the growth of bifidobacteria in a simulated infant microbiota experiment. The slight changes in microbiota composition associated with 2´-FL were reflected by the production of short-chain fatty acids and the reduced production of acetate and lactate in the presence of 2´-FL compared with lactose or GOS. The simulations showed differences in 2´-FL fermentation abilities, indicating that 2´-FL fermentation requires specific microbial activity compared with the fermentation of either lactose or GOS.

In conclusion, we propose that xylitol can be considered an active ingredient for the inhibition of planktonic *S. mutans* growth and early biofilm formation. 2´-FL did not support *S. mutans* growth and thus should not promote unfavourable changes in caries microbiota. Finally, the infant colon and dental simulation models were found to serve as functional models for studying the effects of various food ingredients on bacterial growth, metabolite production and biofilm formation.

KEYWORDS: *Streptococcus mutans*, oral microbiota, adherence, biofilm, infant colonic microbiota, human milk oligosaccharides, xylitol, 2´-fucosyllactose, galacto-oligosaccharides

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Hammaslääketieteen laitos
Parodontologia
KRISTA SALLI: Suu- ja suolistomikrobiston muokkaus ksylitolilla ja 2´-fukosyylilaktoosilla: *in vitro* tutkimukset
Väitöskirja, 139 s.
Suun terveystieteiden tohtoriohjelma – FINDOS Turku
Marraskuu 2020

TIIVISTELMÄ

Ruuansulatus alkaa suusta ja jatkuu ruuansulatuskanavan kautta paksusuoleen. Sekä suussa että suolistossa on tiheä bakteeristo. Mikrobiston koostumusta voi muovata eri ruoka-ainella, kuten sokerilla, ksylitolilla ja äidinmaidon oligosakkarideilla (HMO:lla). Tutkimuksen tavoitteina oli arvioida 1) ksylitolin ja yhden HMO:n 2´-fukosyylilaktoosin (2´-FL:n) vaikutusta hampaiden reikiintymiseen kytkeytyvän *Streptococcus mutans* -bakteerin kasvuun, biofilmin muodostumiseen ja kiinnittymiseen pinnoille ja 2) tutkia 2´-FL:n vaikutuksia mallinnettuun vauvan suolistomikrobiston koostumukseen ja sen tuottamiin aineenvaihduntatuotteisiin. Tutkimuksessa käytettiin suuta ja suolistoa jäljitteleviä *in vitro* malleja.

Mutans streptokokkien biofilmin muodostus väheni ksylitolilla ja ksylitoliminttupastilleilla ja lisääntyi sakkaroosilla ja sakkaroosipohjaisilla minttupastilleilla. *S. mutans* kasvoi hyvin galakto-oligosakkarideilla (GOS:lla), mutta ei 2´-FL:lla ja ksylitol hidasti sen kasvua. Kiinnittymiskokeissa ei löydetty 2´-FL:lle ja GOS:lle samansuuntaista vaikutusta ja 1% ksylitoli ei estänyt *S. mutans* bakteerin kiinnittymistä. 2´-FL, GOS ja laktoosi, lisäsivät kaikki bifidobakteereiden määrää mallinnetussa vauvan mikrobistossa. Pienet 2´-FL:n aiheuttamat muutokset mikrobistossa näkyivät myös lyhytketjuisten rasvahappojen tuotossa. Mallinnettu mikrobisto muodosti 2´-FL:lla vähemmän asetaattia ja laktaattia kuin GOS:lla ja laktoosilla. Mallinnusten välillä oli eroja 2´-FL n käytössä, mikä viittaa siihen että 2´-FL:n käyttäminen vaatii tarkoin määrätyn mikrobistokoostumuksen verrattuna laktoosiin ja GOS:hin.

Yhteenvetona voidaan todeta, että ksylitolia voidaan pitää aktiivisena ruuan ainesosana, joka hidastaa *S. mutans:n* kasvua ja vähentää biofilmin muodostusta. *S. mutans* ei kasvanut 2´-FL:lla, mikä viittaa siihen, että sen käytöstä ei seuraa haitallisia vaikutuksia suumikrobiston koostumukseen. Suun biofilmin ja vauvan suolistoa jäljittelevät *in vitro* mallit todettiin hyödyllisiksi arvioitaessa ruuan ainesosia, joilla voidaan vaikuttaa mikrobiston koostumukseen.

AVAINSANAT: *Streptococcus mutans*, suun mikrobisto, kiinnittyminen, biofilmi, vauvan suolen mikrobisto, äidinmaidon oligosakkaridit, ksylitoli, 2´-fukosyylilaktoosi, galakto-oligosakkaridi

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Abbreviations

2′-FL	2′-fucosyllactose
AS	artificial saliva
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AUC	area under the growth curve
BCFAs	branched-chain fatty acids
BF	breast-fed
BHI	brain-heart infusion medium
CI	clinical isolate
CoMiniGut	Copenhagen mini gut
DNA	deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen
EPS	extracellular polymeric substance
EFSA	European Food Safety Agency
FDR	false-discovery rate
FF	formula-fed
FOS	fructo-oligosaccharides
Fuc	fucose
Gbp	glucan-binding protein
CCUG	Culture Collection, University of Gothenburg, Sweden
Glu	glucose
Gtf	glucosyltransferase
GOS	galacto-oligosaccharides
HA	hydroxyapatite
HMO	human milk oligosaccharide
HPLC	high-performance liquid chromatography
MS	mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus)*
OD	optical density
PO	peppermint oil
PolyFermS	polyfermentor intestinal model
qPCR	quantitative polymerase chain reaction

SCFAs	short-chain fatty acids
SHIME	simulator of human intestinal ecosystem
TIM	TNO intestinal model
TSB	tryptic soy broth
V_1	vessel 1
V_2	vessel 2
V_3	vessel 3
V_4	vessel 4

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Salli KM, Forssten SD, Lahtinen SJ, Ouwehand AC. Influence of sucrose and xylitol on an early *Streptococcus mutans* biofilm in a dental simulator. *Archives of Oral Biology,* 2016;70:39–46.

II Salli KM, Gürsoy UK, Söderling EM, Ouwehand AC. Effects of Xylitol and Sucrose Mint Products on *Streptococcus mutan*s Colonization in a Dental Simulator Model. *Current Microbiology*, 2017; 74:1153–1159.

III Salli K, Söderling E, Hirvonen J, Gürsoy UK, Ouwehand AC. Influence of 2´-fucosyllactose and galacto-oligosaccharides on the growth and adhesion of *Streptococcus mutans*. *British Journal of Nutrition*, 2020; 124: 824–831.

IV Salli K, Anglenius H, Hirvonen J, Hibberd AA, Ahonen I, Saarinen MT, Tiihonen K, Maukonen J, Ouwehand AC. The effect of 2'-fucosyllactose on simulated infant gut microbiome and metabolites; a pilot study in comparison to GOS and lactose. *Scientific Reports,* 2019; 9:13232.

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1 Introduction

The oral cavity represents the first section of the gastrointestinal tract, extending from the lips and cheeks and continuing into the oropharynx. The oral cavity is composed of both hard (teeth and jaws) and soft tissues (lips, gingiva, oral mucosa and tongue, Fig. 1). The primary function of the oral cavity involves the ingestion of food, including mastication and swallowing. Other functions include speech and ventilation.

The colon represents the end of the gastrointestinal tract. (Fig. 1). The primary components of the colon include the cecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum. The primary physiological functions of the colon include the absorption of water and salts and the formation and storage of faecal material (Carrington, 2014). Moreover, microbiota in the colon perform many essential functions, which are described in more detail below.

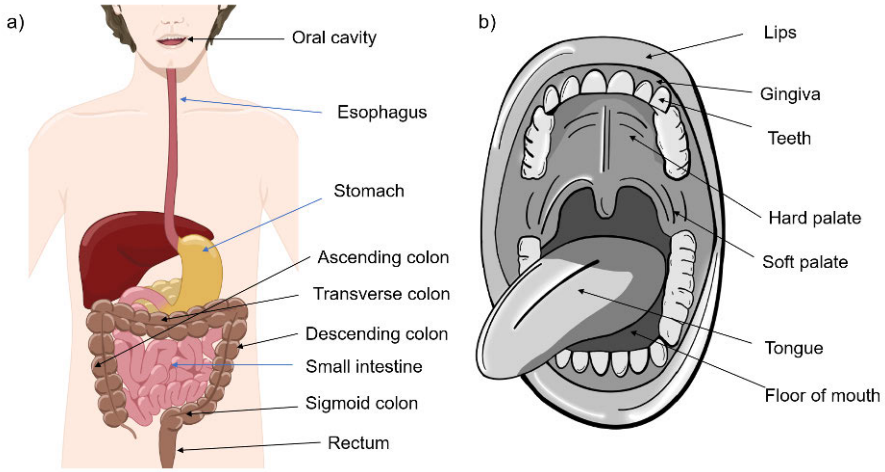

Figure 1. a) The gastrointestinal tract, starting from the oral cavity and ending in the colon (Copyright Pinja Kettunen/SciArt and DuPont Nutrition & Biosciences, with permission). b) The oral cavity (own drawing).

This thesis describes the study of two rich microbial environments: the oral cavity and the colon (Huttenhower *et al.*, 2012). Currently, humans are estimated to carry 1.3-fold more bacterial cells than human cells (Sender *et al.*, 2016); therefore, commensal bacteria likely play many important functions, which are specific to their habitats. Diet is crucial for the healthy development and function of both oral and infant gut microbiota communities, and any disruptions in the quality or quantity of diet have major consequences on microbial composition and virulence. The frequent intake of fermentable carbohydrates and the subsequent (local) acid production can shift the balance of oral microbiota away from a symbiotic relationship between commensal oral microbes and the host (Marsh, 2018). This dysbiosis is characterised by the production of extracellular polymeric compounds and acidic metabolic products, which favours the proliferation of acidogenic and aciduric organisms, such as *Streptococcus mutans* (Lamont *et al.*, 2018; Marsh, 2018). Adjustments in diet, such as the habitual consumption of xylitol, may prevent the development of dysbiosis associated with a sucrose-containing diet and snacking (Söderling *et al.*, 2015).

The gut microbiota develops during infancy, and interactions between microbes and the host during this period can have long-lasting consequences (Milani *et al.*, 2017). Breast milk is often the primary food source for infants. When breastfeeding is not possible, formula provides an alternative food source. Because the composition of breast milk changes during lactation and varies interindividually, formula represents a compromise, in both composition and function. Breast milk contains many bioactive molecules, including human milk oligosaccharides (HMOs), a collection of over 150 different structures, which are not digested by infants but can nourish and alter the infant microbiota composition because only certain bacteria are able to utilise them as an energy source (Li *et al.*, 2020; Milani *et al.*, 2017; Urashima *et al.*, 2018). HMOs can be considered an infant's first prebiotics. During adulthood, microbiota diversity and well-being can be augmented by the provision of complex carbohydrates in the diet (Sonnenburg & Bäckhed, 2016). The fermentation of these compounds results in the generation of metabolites, such as short-chain fatty acids (SCFAs), which have beneficial effects on the human host (Li *et al.*, 2020; Sonnenburg & Bäckhed, 2016).

In vitro methods can be used to study how different compounds affect bacterial characteristics. Studying various factors requires different approaches. Bacterial characteristics differ between the planktonic state and biofilms. In addition, environmental factors such as atmospheric gas, pH, the complexity of the bacterial consortia and bacterial density can also affect the characteristics of both specific bacteria and the broader bacterial community. Model systems are especially important when studying colonic fermentation. The sampling from colon is invasive; thus, various *in vitro* models of colon or other parts of gastrointestinal tract have been developed (Dupont *et al.*, 2019; Pham & Mohajeri, 2018) and widely used to study the effects of food components, pre-, pro- and synbiotics on microbiota.

2 Review of the Literature

2.1 Oral microbiota

2.1.1 Introduction

The oral cavity has very a rich microbiota, with only the colonic microbiota being more diverse (Huttenhower *et al.*, 2012). Distinct bacteria preferentially populate different sites of the oral cavity, including the hard enamel surface and the soft epithelial, palate, vestibule and tongue tissues (Fig. 1b). Preferential colonisation depends on the adhesins found on bacterial membranes and their matching counterparts on specific oral surfaces, in addition to other environmental factors (Aas *et al.*, 2005; Kolenbrander *et al.*, 2010). In addition to bacteria, viruses, fungi, archaea and protozoa can also be found in the oral cavity, although they are currently less studied (Rosier *et al.*, 2018; Wade, 2013; Zhang *et al.*, 2018).

Bacteria in the oral cavity exist in both planktonic (in saliva) and biofilm (dental plaque) forms. Bacterial numbers have been estimated to be approximately 10^{11} bacteria/ml in dental plaque and 10^{9} bacteria/ml in saliva (Sender *et al.*, 2016). Biofilms represent a matrix of bacteria and exocellular polysaccharides, which alters many important bacterial physiological properties (Lamont *et al.*, 2018; Marsh, 2012); for example, both 3-day-old and 3-h-old *S. mutans* cells in biofilms were much more resistant to acid stress than the same bacteria in the planktonic form (Welin-Neilands & Svensäter, 2007; Welin *et al.*, 2003). In biofilms, bacterial cells reside in close proximity to each other, communicating through quorum sensing, competing for resources and gaining protection from antimicrobials and shear forces (Bowen *et al.*, 2018; Li & Tian, 2012). The environment within the biofilm is variable, with gradients of oxygen, pH and nutrients (Lamont *et al.*, 2018).

Different host factors can influence the compositions of oral microbiota, including ageing, genetics, lifestyle (together with diet), medications and environmental factors [reviewed in (Cornejo Ulloa *et al.*, 2019)]. Under healthy state conditions, the bacteria in the oral cavity live in symbiosis with their human host, preventing the invasion of pathogens and providing benefits to host physiology, metabolism and immune function (Cornejo Ulloa *et al.*, 2019; Kilian *et al.*, 2016; Marsh, 2018). This delicate balance between the host and the microbiota in the oral cavity can lead to mutual

benefits. In contrast, in disease states (such as caries or periodontitis), dysbiosis disrupts the host-microbiota balance (Lamont *et al.*, 2018; Rosier *et al.*, 2018; Wade, 2013). Different hypotheses have been proposed regarding the relationship between bacterial biofilms and dental diseases. For periodontal disease, complex interactions between host immune factors and bacterial biofilms are required for gingivitis to progress to periodontitis (Lamont *et al.*, 2018; Zhang *et al.*, 2018). Thus, changes in the composition of the bacterial biofilm and the dysregulation of the host inflammatory response are important for disease development (Lamont *et al.*, 2018). The frequent intake of dietary carbohydrates can shift the microbiota composition to favour acidogenic and aciduric species, promoting the development of caries (Lamont *et al.*, 2018; Tanner *et al.*, 2018; Zhang *et al.*, 2018). The mechanical removal of bacterial biofilms, antibiotics, antimicrobials, probiotics, prebiotics and bacteriophages have all been used to modulate the microbiota composition and shift the balance from dysbiosis back to homeostasis (Zhang *et al.*, 2018). Overall, contemporary research has focused on microbial balance at an ecological level, rather than on individual bacterial species. This thesis focused primarily on caries microbiota and, more specifically, on the initial adhesion, colonisation, and formation of supragingival biofilms, which refers to the biofilm found on the enamel surface that is more commonly associated with caries.

2.1.2 Colonisation of the oral cavity *in vivo*

The oral cavity presents a moist, warm and nutrient-rich environment for bacteria to inhabit, with various oxygen concentrations (Kilian *et al.*, 2016; Marsh, 2018). More than 700 bacterial species have been identified in the human oral cavity; however, the number of bacterial species from any given individual mouth has been estimated to range between 40 and 200 (Aas *et al.*, 2005; Marsh, 2018). Wide interindividual variance exists in the bacterial compositions among healthy individuals. The oral cavity is subject to temporal changes in the environment, and bacteria must be able to tolerate temperature changes and times of both nutritional abundance and famine (Kolenbrander *et al.*, 2010). The established microbiotas are surprisingly resilient to change, possibly associated with salivary and gingival crevicular fluid flow, which constantly provide nutrients and minerals and neutralise environmental stressors (Rosier *et al.*, 2018).

Early colonisation of the oral cavity during infancy is not yet a well-understood process. Although controversial results have been reported regarding whether bacteria are present *in utero*, recent studies have suggested that oral cavity colonisation may begin that early (Nuriel-Ohayon *et al.*, 2016; Tuominen *et al.*, 2019). The mode of delivery (vaginal birth or caesarean section) and early feeding (breast milk and/or formula) affect also oral cavity colonisation (Al-Shehri *et al.*, 2016; Holgerson *et al.*, 2013). *Streptococcus, Propionibacterium* and *Lactobacillus* (according to the current, new nomenclature introduced by Zheng *et al.,* 2020) *Ligilactobacillus,*

Lacticaseibacillus, Limosilactobacillus and *Latilactobacillus* are among the earliest bacteria found in the oral cavities of neonates (Drell *et al.*, 2017; Nelun Barfod *et al.*, 2011; Tuominen *et al.*, 2019; Zheng *et al.*, 2020). The eruption of teeth alters the oral microbiota of infants, providing hard surfaces for bacteria to attach to (Kilian *et al.*, 2016). However, mutans streptococci (MS, *S. mutans* and *Streptococcus sobrinus)* and lactobacilli can be detected in the infant oral cavity even before teeth erupt (Nelun Barfod *et al.*, 2011; Plonka *et al.*, 2012). Vertical transmission, from mothers or other caregivers to infants, represents the most common acquisition route for MS, but horizontal transfer from siblings or other children, such as in day-care settings, can also occur (Berkowitz, 2006; da Silva Bastos Vde *et al.*, 2015). High maternal MS levels increase the risk of early MS transmission, but also other factors like mode of delivery affect (Berkowitz, 2006). Preventing the early colonisation of MS can maintain good oral health in infancy and can have long-lasting benefits by preventing caries (Köhler & Andréen, 2012; Xiao *et al.*, 2019).

As the infant grows, the oral microbiota diversity increases (Dzidic *et al.*, 2018; Lif Holgerson *et al.*, 2015). The factors that affect the development of oral microbiota in childhood were recently studied by following children from age 3 months until 7 years and characterising their salivary microbiota (Dzidic *et al.*, 2018). Although the mode of delivery affected the microbiota composition during the early stages, the differences vanished as the children aged; however, antibiotic treatments and breastfeeding had long-lasting effects on bacterial composition (Dzidic *et al.*, 2018).

2.1.3 Caries microbiota

Under healthy conditions, the supragingival biofilm, which is found above the gum line, is primarily composed of *Streptococcus, Capnocytophaga, Corynebacterium, Veillonella, Rothia, Actinomyces,* unclassified Pasteurellaceae, unclassified Neisseriaceae and *Fusobacterium* (Keijser *et al.*, 2008; Li *et al.*, 2013). In caries, the compositions of supragingival microbiota changes, and the compositions can also vary across different parts of the enamel and during different phases of lesion development (Jiang *et al.*, 2014; Xu *et al.*, 2018). *S. mutans*, *S. sobrinus* and various lactobacilli have long been known as caries-related pathogens; however, other acid-tolerant bacteria can also impact caries, including non-mutans streptococci, *Actinomyces, Bifidobacterium* and *Scardovia wiggsiae* (Aas *et al.*, 2008; Takahashi & Nyvad, 2011; Tanner *et al.*, 2018; Zhang *et al.*, 2018). Changes in the bacterial composition are accompanied by an increase in active carbohydrate metabolism in both caries active adolescents and children with early childhood caries (Wang *et al.*, 2019; Xu *et al.*, 2018).

Saliva is an easily accessible fluid and can provide an estimate of the overall bacterial composition in the oral cavity. The salivary microbiota composition is somewhat similar

to the microbiota composition of the soft tissues (mucosa, tonsils and tongue) in the oral cavity, consisting of *Prevotella, Streptococcus, Veillonella*, *Fusobacterium,* unclassified Pasteurellaceae, *Porphyromonas* and *Neisseria* (Keijser *et al.*, 2008; Li *et al.*, 2013; Zhang *et al.*, 2018). However, high salivary MS levels correlate with the severity of early childhood caries (Liu *et al.*, 2019). In addition, comparisons of the microbiota compositions found in adolescents with and without caries showed clear differences, with *S. wiggsiae*, *S. mutans* and *Bifidobacterium longum* being more abundant in the saliva samples of participants with caries (Eriksson *et al.*, 2017).

2.1.4 Bacterial adhesion

Mechanical cleaning and the removal of biofilm is essential for dental health. However, the adherence of salivary components to oral surfaces begins immediately after they are cleaned. Bacterial adhesion and colonisation are a well-orchestrated process.

First, an acquired salivary pellicle is formed on the cleaned enamel. This process begins when components with a high affinity for hydroxyapatite (such as proteins statherin, histatins and proline-rich-proteins) bind directly to the enamel, through van der Waal's forces, dipole-dipole effects and hydrophobic interactions (Hannig & Hannig, 2009) and continues with other proteins and glycoproteins including mucins, amylase, lactoferrin, lysozyme and cystatins. Protein-protein interactions are important for pellicle formation, and the host contributes to the intrinsic maturation of the pellicle providing enzymes that react with salivary proteins. The sources of pellicle proteins include salivary gland secretions, gingival crevicular fluid, oral epithelial cell products and micro-organisms. [Reviewed in (Siqueira *et al.*, 2012)]

Oral bacteria colonise the tooth surface by recognising receptors within an acquired enamel pellicle that match their surface adhesin molecules, in addition non-specific mechanisms, such as longer-range interactions and surface roughness affect colonisation (Fig. 2a) (Hannig & Hannig, 2009; Marsh *et al.*, 2016; Nobbs *et al.*, 2009; Siqueira *et al.*, 2012). Therefore, pellicle proteins, other components, and the overall bacterial composition can influence bacterial adherence (Marsh *et al.*, 2016). Initial colonisers, such as *Streptococcus sanguinis, Streptococcus gordonii, Streptococcus oralis, Streptococcus mitis* and *Actinomyces* species, tend to have weak interactions with pellicle components (Nobbs *et al.*, 2009). Subsequently, some bacteria begin to bind more firmly to receptors on the salivary pellicle (Fig. 2b) (Nobbs *et al.*, 2009). These bacteria then provide adhesion sites for further bacteria to co-adhere, forming a biofilm (Kolenbrander *et al.*, 2010). The specificity of bacterial adherence is often related to protein-carbohydrate recognition. Common carbohydrates include galactose, N-acetyl-galactosamine, sialic acid (neuraminic acid), fucose (Fuc), N-acetyl-glucosamine and glucose (Glu) (Nobbs *et al.*, 2009). Because colonisation is a necessary first step for biofilm formation, the prevention

of colonisation, using anti-adhesive compounds, represents an important area of research, which is relevant to the study of caries and periodontal diseases.

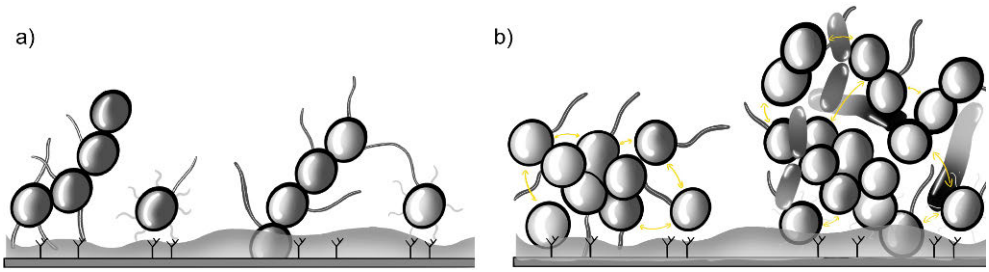

Figure 2. The adherence and colonisation of surfaces in the oral cavity. **a)** Initial colonisers, such as *Streptococcus* spp. approach a conditioned surface and adhere to it, using longer-range interactions (e.g. pili penetrating mucus) or shorter-range interactions (e.g. surface molecules and bacterial adhesins). **b)** Bacteria adapt to different environments, multiply, and interact with each other (arrows). Extracellular polymeric substances are produced and other bacterial species adhere, increasing the bacterial concentration and forming a community [own drawing, modified from (Nobbs *et al.,* 2009)].

2.1.5 Dental biofilm formation

After an acquired enamel pellicle has formed and primary colonisers have adhered to it, the process of biofilm formation continues. However, adhered bacteria require an appropriate environment that supports growth on the attached surface, including the availability of nutrients and appropriate pH, oxygen concentrations, and redox potential (Nobbs *et al.*, 2009). Under suitable conditions, the initial colonisers will begin to multiply and begin to form a small community (Nobbs *et al.*, 2009). Increased bacterial numbers promote the occurrence of interactions and provide new surfaces for other bacteria to bind (Nobbs *et al.*, 2009). Interspecies communication is important and has been linked to the cell-cell proximity between bacteria in the biofilm (Kolenbrander *et al.*, 2010).

Another important factor for biofilm formation is the production of extracellular polymeric substances (EPS). EPS are composed of (exo)polysaccharides and proteins but can also contain lipids and extracellular DNA (Di Martino, 2018; Klein *et al.*, 2015). Exopolysaccharides, especially glucan- and fructan-based polysaccharides, are produced by oral streptococci and contribute to the biofilm matrix (Bowen & Koo, 2011; Nobbs *et al.*, 2009). EPS provide binding sites for bacterial adherence, retain the bacteria in the biofilm and form a polymeric matrix that provides protection, stability and nutrients (Fig. 2 and Fig. 3) (Flemming *et al.*, 2016; Koo *et al.*, 2017; Lamont *et al.*, 2018). As the biofilm matures, the EPS provide gradients of habitable zones, consisting of various concentrations of nutrients, oxygen and pH, enabling more bacteria to grow (Flemming *et al.*, 2016; Koo *et al.*, 2017). Dietary sucrose provides substrates for EPS production and increases acid production by oral bacteria (Bowen *et al.*, 2018).

Biofilm formation contributes to a healthy oral ecosystem. In a healthy individual, biofilm formation does not harm the host if proper oral hygiene is maintained to inhibit excessive bacterial accumulation. The extended ecological plaque hypothesis describes how bacteria in the oral cavity exist in a symbiotic relationship with the host under normal conditions; however, when conditions change, dynamic adaptations can shift the balance in a detrimental direction (Takahashi & Nyvad, 2011). In caries, the frequent consumption of carbohydrates can trigger a change from normal conditions. Oral bacteria can utilise carbohydrates to produce acids locally within a biofilm on the enamel, causing enamel demineralisation and the slow formation of cavities.

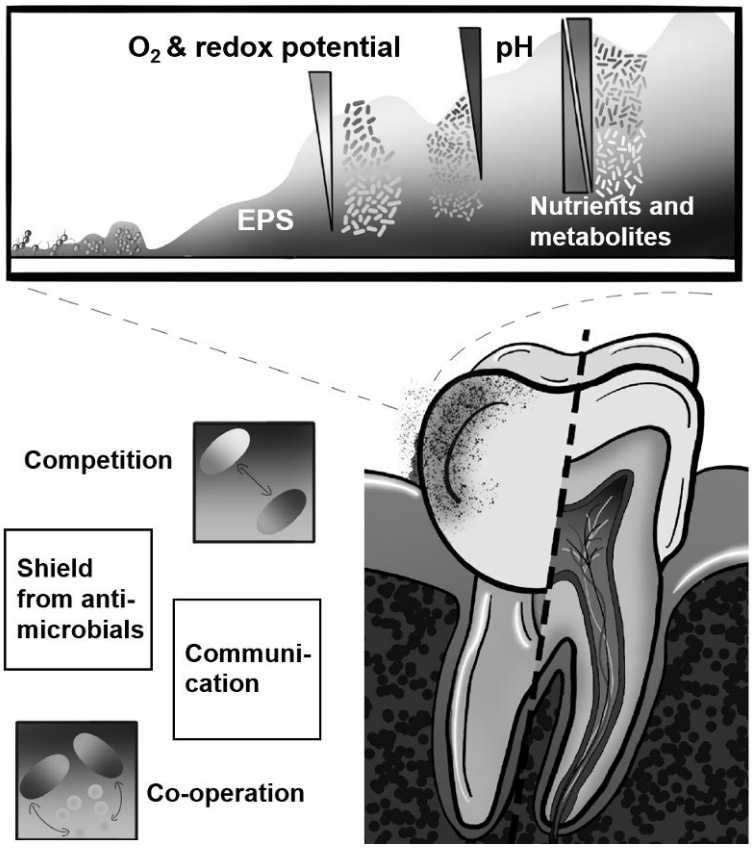

Figure 3. The formation of biofilms on the tooth surface. Increased bacterial numbers and the production of extracellular polymeric substance (EPS), especially after sucrose consumption, contributes to biofilm growth. This biofilm matrix provides protection and stability and forms gradients of habitable zones featuring various concentrations of nutrients, oxygen and pH. Bacteria that are able to thrive in biofilm-form alter many of their characteristics [own drawing, modified from (Bowen *et al.*, 2018; Lamont *et al.*, 2018)].

2.1.6 *Streptococcus mutans*

S. mutans is one of the most well-studied bacteria in relation to caries (Loesche, 1986; Tanzer *et al.*, 2001). Although caries can occur in the absence of *S. mutans,* this species features many capabilities that increase its cariogenic potential, facilitating its role as a key player in caries pathogenesis. *S. mutans* can synthesise large quantities of extracellular polymers and develop the matrix for biofilm growth (Lemos *et al.*, 2019). *S. mutans* can also produce acids efficiently from many carbon sources, and it thrives in acidic conditions (Lemos *et al.*, 2019).

S. mutans produces glucan-binding proteins (Gbps), which contribute to its abilities to build a biofilm matrix (Lemos *et al.*, 2019). In addition, *S. mutans* produces three types of glycosyltransferases (Gtfs)—GtfB, GtfC and GtfD—each of which synthesises different extracellular polysaccharide glucans from sucrose and other sources (Bowen & Koo, 2011; Koo *et al.*, 2010). Secreted Gtfs bind to the salivary pellicle and help other bacteria adhere to the biofilm (Abranches *et al.*, 2018). Gtfs also promote the aggregation and co-aggregation of oral bacteria (Bedoya-Correa *et al.*, 2019). Thus, both Gtfs and Gbps function as components of sucrose-dependent adhesion and contribute to the formation of the biofilm matrix (Bowen & Koo, 2011). Surface adherence can also be mediated through various high-affinity (sucrose-independent) adhesin molecules, which interact with substrates in the acquired enamel pellicle (Abranches *et al.*, 2018; Lemos *et al.*, 2019).

In addition to efficient adherence to the enamel and the production of exopolysaccharides, *S. mutans* also generates organic acids and ferments various carbohydrates (Abranches *et al.*, 2018; Bedoya-Correa *et al.*, 2019). This low-pH (micro-) environment may be harmful to some other bacterial species [e.g. non-mutans streptococci and Actinobacteria (Takahashi & Nyvad, 2011)] and promotes the growth of bacteria that can tolerate acidic conditions (Lamont *et al.*, 2018). *S. mutans* is one of the most acidogenic species found in dental biofilms, with a high rate of acid production and the ability to rapidly adapt to alterations in pH condition (Bedoya-Correa *et al.*, 2019; de Soet *et al.*, 2000; Welin-Neilands & Svensäter, 2007).

S. mutans can also compete and survive in the oral cavity through the production of a variety of bacteriocins (Huang *et al.*, 2018; Merritt & Qi, 2012), which are short peptides that can inhibit the growth or kill closely related bacteria (Merritt & Qi, 2012). Bacteriocins produced by *S. mutans*, are called mutacins (Merritt & Qi, 2012). Mutacin production depends on many factors, including cell density, nutrient sources and oxygen levels (Merritt & Qi, 2012), and mutacins serve to improve the competitiveness of *S. mutans.* As with other bacteria, strain-dependent differences in the virulence of *S. mutans* strains exist (Bedoya-Correa *et al.*, 2019; Lemos *et al.*, 2019).

2.2 Microbiota of the colon

2.2.1 Introduction

The microbiota composition varies across different sections of the gastrointestinal tract, due to differing conditions. The colon features the highest bacterial density, which is currently estimated to be approximately 10^{11} bacteria/g (Sender *et al.*, 2016). In addition to bacteria, the colon contains archaea, viruses, fungi and eukarya (Hillman *et al.*, 2017). The colon environment is characterised by low levels of oxygen, a near-neutral pH and a quite constant level of complex polysaccharides, which bacteria utilise for energy (Donaldson *et al.*, 2016; Evans *et al.*, 1988; Pereira & Berry, 2017). The dominant bacterial phyla include Bacteroidetes and Firmicutes, together with Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia (Donaldson *et al.*, 2016; Rinninella *et al.*, 2019).

Bacteria and the human host have a mutualistic relationship, and the highly complex colonic microbiota performs many functions that benefit the host metabolism, immunity, development and behaviour (McBurney *et al.*, 2019; Woloszynek *et al.*, 2016). High microbial diversity has also been linked to the microbiota resilience, which refers to its ability to return to its original state following perturbations and is a key factor associated with healthy microbiota (McBurney *et al.*, 2019).

Fibres and other food products that are not digested and absorbed by the upper gastrointestinal tract arrive at the colon, where they are digested into absorbable nutrients by various micro-organisms. In a healthy state, the host generally benefits from bacterial metabolic products, and the microbiota population remains relatively stable, assuming that diet, environment, medications, and health state do not change (McBurney *et al.*, 2019). Thus, in a healthy state, following the development of the microbiota community during infancy, some subtle changes will occur during childhood, and then change occur again during older ages (Nuriel-Ohayon *et al.*, 2016).

2.2.2 Composition of colonic microbiota

The microbial composition and diversity can vary in the same individual and between individuals at various times. Many other factors can contribute to the composition of microbiota, including age, weight, lifestyle, diet, diseases, medications, immune function and ethnicity (Donaldson *et al.*, 2016; Rinninella *et al.*, 2019). Similarly, the compositions of colonic microbiota vary among different niches in the colon. Antimicrobials, dietary nutrients, pH, oxygen levels and the level of host immune activity can all shape the composition of microbial populations and

can vary along the length of the colon (Donaldson *et al.*, 2016). The mucus layer, which surrounds crypts in the lumen, creates a boundary between the host epithelium and the gut lumen (Donaldson *et al.*, 2016). Some bacterial species, such as *Akkermansia muciniphila* and *Bacteroides fragilis,* can degrade and utilise mucin glycans, generating distinct habitats near the host epithelium and gaining a competitive advantage (Donaldson *et al.*, 2016).

Early infant microbiota colonisation is important and can affect immune and metabolic maturation. Elements that disrupt healthy colonisation can, therefore, have long-lasting effects, including on the development of diseases (Rautava *et al.*, 2012).

2.2.3 Infant colonic microbiota

Until quite recently, the placenta and the developing foetus were thought to be sterile; however, several studies have reported that bacterial colonisation may begin *in utero* (Collado *et al.*, 2016; Nuriel-Ohayon *et al.*, 2016). Collado *et al.* showed that the amniotic fluid and the placenta have distinct microbiota populations, which may indicate infant gut colonisation, *in utero* (Collado *et al.*, 2016). The maternal microbiota composition changes during pregnancy and may affect infant microbiota development, and interactions between maternal microbes or microbial components and the foetus have been described (Nuriel-Ohayon *et al.*, 2016). However, the first major bacterial exposure for an infant occurs at birth (Fig. 4). During the first months of life, the developing microbiota composition undergoes many short-term changes, and interpersonal variation during this stage of life is greater than in adults (Koenig *et al.*, 2011; Nuriel-Ohayon *et al.*, 2016). Gestational age and the mode of delivery are key factors that affect the composition of the infant microbiota (Rinninella *et al.*, 2019). The microbiota of preterm infants is less diverse and is characterised by the increased colonisation of potentially pathogenic species compared with full-term infants (Rinninella *et al.*, 2019).

Vaginally delivered infants are colonised first by bacterial species from the mother's vagina and gut, such as *Lactobacillus, Bifidobacterium, Bacteroides, Prevotella, Enterococcus, Escherichia, Streptococcus* and *Rothia* (Bäckhed *et al.*, 2015; Nuriel-Ohayon *et al.*, 2016; Zhuang *et al.*, 2019). Infants born by caesarean section feature a gut microbiota characterised by bacterial species from the maternal skin and oral cavities, in addition to bacteria from the surrounding environment, such as *Staphylococcus, Propionibacterium, Corynebacterium* and *Streptococcus* (Fig. 4) (Bäckhed *et al.*, 2015; Nuriel-Ohayon *et al.*, 2016; Rinninella *et al.*, 2019; Vandenplas *et al.*, 2018). The prevalence of bifidobacteria increases in infants over time, regardless of the mode of birth (Bäckhed *et al.*, 2015; Zhuang *et al.*, 2019).

Mothers appear to represent an important source of early colonisers for the infant microbiota (Bäckhed *et al.*, 2015). The early feeding mode is another important factor (Fig. 4). Human breast milk features its own microbiota, which affects the development of an infant's gut microbiota (Nuriel-Ohayon *et al.*, 2016). Breast milk contains multiple bioactive components, including nutrients and immunomodulatory molecules, and the composition of breast milk changes throughout lactation (Chong *et al.*, 2018; Moore & Townsend, 2019). HMOs are the third-most-abundant components in human milk (after lactose and lipids), passing through the infant digestive system mostly intact to feed the developing microbiota (Bode, 2012). Some infant bacteria, including certain *Bifidobacterium, Bacteroides* and *Lactobacillus* (according to the current, new nomenclature introduced by Zheng *et al.,* 2020) *Lactobacillus* and *Lactiplantibacillus,* are able to digest HMOs (Bode, 2012; Nuriel-Ohayon *et al.*, 2016; Thongaram *et al.*, 2017; Zheng *et al.*, 2020; Zúñiga *et al.*, 2018); thus, breast-fed infants commonly present greater numbers of *Bifidobacterium* spp. than infants fed with a non-prebiotic formula (Rinninella *et al.*, 2019). HMOs also have other functions, which are discussed in more detail in Section 2.3.2.2.

The microbiota of formula-fed infants tends to be more diverse than that of breast-fed infants, dominated by enterococci and clostridia, whereas in breast-fed infants bifidobacteria are abundant (Bezirtzoglou *et al.*, 2011; Chong *et al.*, 2018; Nuriel-Ohayon *et al.*, 2016). The next key event is the introduction of solid foods to provide nutrition to an infant. This event increases bacterial diversity and changes the infant microbiota to more resemble that in an adult, featuring bacterial species such as *Clostridium*, *Ruminococcus*, *Faecalibacterium*, *Roseburia* and *Anaerostipes* (Bäckhed *et al.*, 2015; Zhuang *et al.*, 2019). The initial bacterial colonisation process is very important to both the development and maturation of the gastrointestinal tract (Wampach *et al.*, 2017). Likewise, it can influence infant development and the maturation of the immune system and creates a niche in which adult-like microbiota can later grow (Zhuang *et al.*, 2019). However, many environmental factors including siblings, farm animals, pets, geographical location, genetics, medications, disease and city or countryside living can affect microbiota compositions (Fig. 4) (Nuriel-Ohayon *et al.*, 2016; Zhuang *et al.*, 2019). The composition of the microbiota determines the metabolite profiles produced by these complex communities.

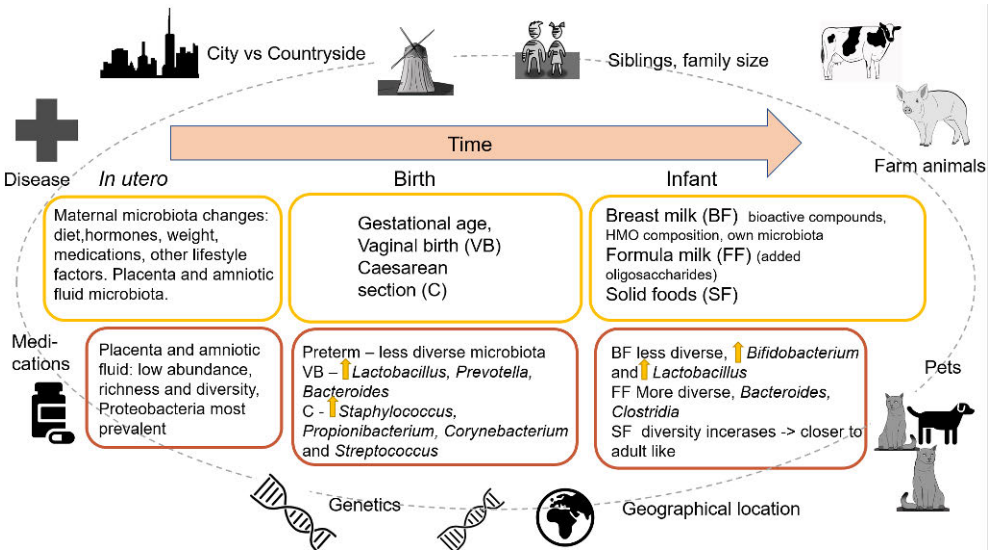

Figure 4. Some factors that influence infant microbiota during development [own drawing, information based on (Chong *et al.*, 2018; Nuriel-Ohayon *et al.*, 2016; Zhuang *et al.*, 2019)].

2.2.4 Metabolites of the microbiota

The composition of the microbiota is one aspect, but another is the combined activity of the bacteria; which metabolites they produce, communally. Undigested fibres serve as an energy source for bacteria and the fermentation process results in the production of SCFAs and other metabolites. This increase the energy obtained from food (Sonnenburg & Bäckhed, 2016; Woloszynek *et al.*, 2016). Colonic bacteria contribute to vitamin synthesis (especially vitamins B and K), and the metabolites produced by microbiota influence intestinal health and immune functions, even during infancy (Dominguez-Bello *et al.*, 2019; LeBlanc *et al.*, 2017; Rowland *et al.*, 2018). In the complex bacterial community, the metabolites produced by some bacteria can serve as food for others, and the carbohydrates that are broken down by some bacteria can be further metabolised by others, in a process referred to as cross-feeding (Ríos-Covián *et al.*, 2016; Rowland *et al.*, 2018). Therefore, some bacterial metabolites are used by other members of the colonic bacterial community, others are absorbed into the host's circulation, and the remainder is excreted in faeces and urine (Sonnenburg & Bäckhed, 2016). In pure culture experiments, the metabolites produced by a single bacterial strain can be evaluated; however, in a complex consortium, how any specific bacterial species affect measurable metabolites can only be speculated. SCFAs (acetate, propionate and butyrate) are produced in the colon, primarily by bacterial carbohydrate fermentation, and are efficiently absorbed by the host (Macfarlane & Macfarlane, 2003; Ríos-Covián *et al.*, 2016). Many

bacterial species, including lactobacilli and bifidobacteria, also produce lactate, which is not an SCFA (Ríos-Covián *et al.*, 2016) but is typically further metabolised, through cross-feeding, into SCFAs. SCFAs, especially butyrate, provide energy (adenosine triphosphate, ATP) to epithelial cells (colonocytes), whereas acetate and propionate are further metabolised by the liver (Ríos-Covián *et al.*, 2016). Propionate is integrated into gluconeogenesis, whereas acetate and butyrate are integrated into lipid synthesis (Ríos-Covián *et al.*, 2016; Sonnenburg & Bäckhed, 2016). SCFAs also lower the luminal pH and may contribute to protection from bacterial pathogens and viral infections (Ríos-Covián *et al.*, 2016). SCFAs have also been shown to promote infant intestinal health and growth (Jacobi & Odle, 2012).

Dietary and host-derived proteins represent important energy sources for bacteria, especially when carbohydrates are scarce, which may be the case in the distal colon. Proteins and peptides are hydrolysed by bacteria and pancreatic proteases, and their fermentation contributes to the production of SCFAs, N-nitroso compounds, phenolic and indolic compounds, branched chain fatty acids (BCFAs), biogenic amines, hydrogen sulphite and carbon dioxide (Yao *et al.*, 2016). These metabolites are either utilised by other members of the microbiota, absorbed by the host, excreted (in faeces, urine and breath) or detoxified by the epithelium (Yao *et al.*, 2016). Some of these proteolytic fermentation products may potentially be harmful to the host, especially if luminal concentrations increase; thus, carbohydrates are considered to represent the preferred energy source for gut bacteria (Yao *et al.*, 2016). Current knowledge regarding the BCFAs levels and functions in infants remains very limited. Longer, 11-26-carbon-chain BCFAs that are synthesised by the normal skin, including vernix caseosa in infants, and can be found in the meconium (Ran-Ressler *et al.*, 2008). Longer BCFAs can also be found in breast milk (Dingess *et al.*, 2017; Jie *et al.*, 2018). Thus, BCFAs can be found in the normal infant gastrointestinal tract and may impact bacterial colonisation (Ran-Ressler *et al.*, 2008; Ran-Ressler *et al.*, 2013).

The microbiota composition of breast-fed infants is largely defined by the constituents of the mother's milk, and the metabolites produced by bacteria enhance adaptive immune response (Dominguez-Bello *et al.*, 2019). The beneficial effects associated with an abundance of bifidobacteria in early life have been suggested to be mediated through the modulation of the immune system and reduction in colonic pH due to the production of SCFAs and lactate (Milani *et al.*, 2017). Changes in the infant diet during the first year of life are associated with changes in the gut bacteria, including adaptations to different carbon sources that are associated with more adult-like microbiota (Bäckhed *et al.*, 2015). The transition to solid foods also results in higher levels of SCFAs (Koenig *et al.*, 2011).

2.3 Modulating oral and gut microbiota

Our understanding of what constitutes healthy microbiota in different parts of the body continues to grow; however, interindividual variations can be large. Aside from environmental and genetic factors, methods have been developed to purposefully enhance the microbiota composition. The oral microbiota is modulated by the composition of the diet, eating frequency, oral hygiene habits, the secretion of saliva, smoking, oral infections and age (Yamashita & Takeshita, 2017). In addition, the use of pro- or prebiotics may modulate the composition of oral microbiota.

To alter the gut microbiota composition and/or function, pro- and prebiotics are commonly used. A 'probiotic' is defined as "live microorganisms that when administered in adequate amounts, confer a health benefit on the host" (Hill *et al.*, 2014). The current definition for the term 'prebiotic' is "a substrate that is selectively utilised by host microorganisms conferring a health benefit" (Gibson *et al.*, 2017)." This new definition has broadened the term prebiotic to include substances other than carbohydrates, areas of the human body other than the gastrointestinal tract and methods for providing prebiotic other than in food (Gibson *et al.*, 2017). In this thesis, I focused on the prebiotic-like effects of an HMO, 2′-fucosyllactose (2′-FL), and xylitol which affect some oral bacteria.

2.3.1 Modulating oral microbiota

Caries is a multifactorial disease, in which changes in the composition of dental biofilm bacteria, diet and other factors can affect pathogenesis (Baker & Edlund, 2018; Philip *et al.*, 2018a). A diet containing fewer fermentable carbohydrates that are consumed less frequently, combined with good oral hygiene, can improve the balance of dysbiotic microbiota. Broad-spectrum antimicrobial agents, including chlorhexidine and triclosan, have also been used to decrease dental biofilms; however, these agents also disrupt beneficial commensal bacteria and can represent a hazard to the environment (Jesus *et al.*, 2013; Olaniyan *et al.*, 2016). Some possibilities for modulating oral microbiota are discussed below.

2.3.1.1 Xylitol

Xylitol is a five-carbon polyol (Fig. 5) that has been studied for its preventive effects against caries, especially against MS. Xylitol is widely used in confectionery (tablets or pastilles and gums) and dental care products, including toothpaste, mouthwashes and varnishes. The beneficial effects of xylitol can be obtained by adding 5–7 g xylitol/day to a normal diet (Mäkinen, 2011; Söderling, 2009). Xylitol stimulates salivary secretion (Mäkinen, 2010), similar to many other sweet products as a non-specific effect. Xylitol has primarily been studied for its effects on MS or *S. mutans*

[reviewed in (Li & Tanner, 2015; Mäkinen, 2011; Söderling, 2009)]. Xylitol has been shown to affect *S. mutans* numbers, both *in vitro* and *in vivo*, as described in more detail below. In addition, xylitol consumption has been shown to decrease dental plaque amounts and acidogenicity (Maguire & Rugg-Gunn, 2003; Splieth *et al.*, 2009; Söderling, 2009; Wennerholm *et al.*, 1994). However, the effects of xylitol on oral bacteria other than MS are not well-known. Studies have indicated a decrease in plaque or salivary MS levels in the presence of xylitol, but no other changes in plaque or salivary bacterial composition have been reported (Söderling *et al.*, 2015; Söderling *et al.*, 2011). A recent systematic review focusing on the effects of xylitol on MS found that xylitol decreased MS levels but did not alter the overall oral microbiota (Söderling & Pienihäkkinen, 2020).

2.3.1.1.1 Overview of clinical studies with xylitol on caries and mutans streptococci

The positive effects of xylitol on dental health were discovered in the Turku sugar studies, performed in the 1970s [I -XVII, overviewed in (Scheinin & Mäkinen, 1976)]. In these studies, the early aim was to replace almost all carbohydrates in the diet with xylitol, fructose and sucrose. After this, the use of xylitol gum as an adjunct to a normal diet was found to provide a similar result and was easier, in practice. Since then, several studies, using different doses and vehicles for xylitol delivery, have evaluated the effects of xylitol, either on caries prevention directly or on cariogenic micro-organisms, such as MS. For practical reasons, many early clinical studies were performed using randomisation by classrooms, not by individual participants. Although these studies were able to recruit large numbers of children, they do not qualify as randomised controlled trials, the current standard for clinical trials. Systematic reviews that have evaluated the efficacy of xylitol for caries prevention have reported evidence that supports xylitol or found no correlation between xylitol use and caries prevention (Deshpande & Jadad, 2008; Newton *et al.*, 2019; Riley *et al.*, 2015). The lack of efficacy in xylitol clinical studies is often associated with a low xylitol dose, sometimes combined with a short study duration or a cohort with low caries prevalence (Mäkinen, 2010). However, in those studies performed on populations in which caries prevalence is high, the preventive effects of xylitol (in adequate doses) has been reliably and repeatedly demonstrated in the literature, verifying the caries-preventive effects of xylitol (Hietala-Lenkkeri, 2016). The European Food Safety Agency (EFSA) has approved the health claim that "xylitol chewing gum reduces the risk of caries in children" (EFSA, 2008).

An important aspect of microbiota modulation is reducing the transmission of MS from mothers (or caregivers) to children. The regular use of xylitol by mothers has been shown to result in a decrease in future caries occurrence of children

[reviewed in (Li & Tanner, 2015; Lin *et al.*, 2016)]. The effects of maternal xylitol use continued to be apparent at the 10-year follow-up, with reduced caries occurrence and a lower need for restorative treatments in children (Laitala *et al.*, 2013).

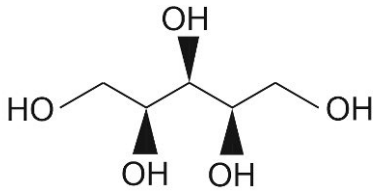

Figure 5. Chemical structure of xylitol (own drawing).

2.3.1.1.2 The effects of xylitol on *Streptococcus mutans* growth and adhesion *in vitro*

To understand the mechanism of action underlying the positive effects of xylitol, several *in vitro* studies have been conducted. Many studies have focused on the growth of MS in the planktonic state because xylitol is non-fermentable by MS, which inhibits bacterial growth (Bradshaw & Marsh, 1994; Söderling *et al.*, 2008; Trahan *et al.*, 1996). Xylitol is incorporated into *S. mutans* via the phosphoenolpyruvate-phosphotransferase system (Miyasawa-Hori *et al.*, 2006; Trahan, 1995). Xylitol-5-phosphate inhibits glycolytic enzymes, and the futile cycle of dephosphorylating xylitol-5-phosphate consumes energy, hindering the growth and acid production of *S. mutans* (Miyasawa-Hori *et al.*, 2006; Trahan, 1995).

More recently, *S. mutans* biofilm formation either in single- or multispecies biofilms, was evaluated. The formation of a 6-species biofilm was inhibited by 1% and 3% xylitol (Badet *et al.*, 2008). However, the intermittent exposure of a 6-species biofilm to 7.5% xylitol failed to affect biofilm growth (Giertsen *et al.*, 2011). In contrast, the young biofilm formation of three species (*S. mutans, S. sanguinis,* and *Actinomyces naeslundii)* was inhibited by 5% xylitol (Marttinen *et al.*, 2012). A very recent study evaluated the biofilm formation dynamics of nine *S. mutans* strains and found that the biofilm formation of all strains was inhibited by 5% xylitol; however, sensitivity to xylitol varied among the strains (Loimaranta *et al.*, 2020). In addition to growth inhibition, xylitol affects biofilm formation by modifying bacterial adherence properties, such as the EPS production of *S. mutans* (Ferreira *et al.*, 2015; Lee *et al.*, 2009; Söderling *et al.*, 1987; Söderling & Hietala-Lenkkeri, 2010). A recent study suggested that xylitol also impairs oral biofilm formation, by inhibiting bacterial β-glucosidase activity in human saliva (Teixeira Essenfelder *et al.*, 2019).

2.3.1.2 Other polyols, probiotics and prebiotics

In addition to xylitol, other polyols have been evaluated according to caries related parameters (Mäkinen, 2011). Sorbitol is consumed by some of the oral bacteria but can be considered cariostatic (Birkhed *et al.*, 1984; Gupta, 2018; Kalfas *et al.*, 1990; Mäkinen, 2011). Maltitol has historically been used as a control, in many xylitol studies, but limited data are available on caries-related parameters (Gupta, 2018; Prosdocimi *et al.*, 2017). Erythritol has demonstrated contradictory effects on the levels of MS (Mäkinen, 2010; Mäkinen *et al.*, 2001; Runnel *et al.*, 2013; Söderling & Hietala-Lenkkeri, 2010) but may be effective for caries prevention; however, the clinical data remain very limited (de Cock *et al.*, 2016). Erythritol is better tolerated in the gastrointestinal tract than sorbitol, maltitol or xylitol (Mäkinen, 2016).

Although not as clear as in the gut, some substances can be regarded as oral prebiotics. Commensal oral bacteria, such as *S. sanguinis* and *S. gordonii* can utilise arginine to produce ammonia, generating higher pH conditions in their surroundings (Baker & Edlund, 2018; Bowen *et al.*, 2018; Philip *et al.*, 2018b). This process also generates ATP, which is beneficial for the commensal bacteria (Bowen *et al.*, 2018).

Lactobacilli and bifidobacteria, which are common probiotics used to target the gut microbiota, have been evaluated for the prevention of caries. Although results have been encouraging, both species are acidogenic and aciduric (Baker & Edlund, 2018; Philip *et al.*, 2018b). However, some strains of lactobacilli and bifidobacteria have demonstrated anti-*S. mutans* effects, such as decreasing adherence and reducing MS counts (Haukioja *et al.*, 2008; Laleman *et al.*, 2014). Among the studies examining caries as an outcome, the current evidence is insufficient (Gruner *et al.*, 2016). Recent probiotics with promising data include *Streptococcus dentisani* and *Streptococcus* A12*,* which are both able to colonise the tooth surface, raise the pH of the dental biofilm and inhibit MS growth (Huang *et al.*, 2016; López-López *et al.*, 2017); however, clinical data are scarce.

Other possible methods to modify oral microbiota include the specific targeting of antimicrobial peptides, bacteriophages and natural products, such as polyphenols from propolis and cranberry, proanthocyanidins and quorum-sensing targets (Baker & Edlund, 2018; Philip *et al.*, 2018b; Zhan, 2018; Zhang *et al.*, 2018).

2.3.2 Modulating gut microbiota

Many diseases, including irritable bowel disease, asthma, allergy, eczema, obesity, neuropsychiatric disorders, necrotising enterocolitis and late-onset sepsis, have been linked to disturbances in the microbiota (Zhuang *et al.*, 2019). Therefore, developing means to modulate the compositions and activity of microbiota is important. Diet represents one of the most impactful methods to regulate microbiota (Woloszynek

et al., 2016). Foods that are rich in plant-based polysaccharides and resistant starches act as bacterial substrates. When foods or prebiotics are fermented by selected bacteria, SCFAs and other metabolites are produced, lowering the luminal pH, providing energy for gut epithelial cells, increasing mucin production, altering metabolite and mineral absorption and enhancing epithelial barrier integrity (Miqdady *et al.*, 2020; Woloszynek *et al.*, 2016). The most common probiotics consist of species from the genera *Bifidobacterium* and *Lactobacillus* (Zhuang *et al.*, 2019). Many probiotic effects are strain-specific (Sánchez *et al.*, 2017). However, some properties may be more general and common among the probiotic strains. Probiotics can normalise disturbed intestinal microbiota compositions, strengthen the gut epithelial barrier, affect SCFA and BCFA production and modulate the immune system, by producing immunomodulatory molecules, including anti-inflammatory cytokines and other antimicrobial substances that prevent pathogen growth through competitive exclusion [reviewed in (Plaza-Diaz *et al.*, 2019)]. This thesis has focused on the prebiotic modifications of microbiota.

2.3.2.1 Prebiotics

One approach used to modify microbiota compositions is the addition of non-digestible nutrients that can only be utilised by specific bacteria. Common prebiotics include various oligosaccharides, such as β (2–1) fructans, inulin, fructo-oligosaccharides (FOS), glucose-based polydextrose and lactulose and galactose-based galacto-oligosaccharides (GOS), all of which can be fermented by colonic bacteria (Ackerman *et al.*, 2017b; Woloszynek *et al.*, 2016; Zhuang *et al.*, 2019). The consumption of prebiotics often stimulates the growth of beneficial bacteria, including bifidobacteria and lactobacilli (Hillman *et al.*, 2017). Other properties of prebiotics include improving specific lipid and mineral metabolism biomarkers, increasing microbial mass, the relief of constipation and the modulation of immune functions (Ouwehand *et al.*, 2005). Metabolites produced by bacterial fermentation, such as SCFAs, can lower the intestinal pH, enhance cellular proliferation, inhibit pathogens and improve mineral availability (Hillman *et al.*, 2017; Ouwehand *et al.*, 2005).

Limited data exist on the effects of xylitol on gut microbiota composition. *In vitro* colon simulations suggest that simulated colonic microbiota can ferment xylitol, and xylitol fermented by the microbiota increased butyrate production compared with control simulations (Mäkeläinen *et al.,* 2007). Animal studies (mice) have associated xylitol consumption with decrease in *Bacteroides,* increase in *Prevotella* and suppression of *Candida* [reviewed in (Salli *et al.*, 2019)].

2.3.2.2 Human milk oligosaccharides

HMOs represent a diverse group of oligosaccharides found in human milk and can be considered to be first prebiotics for infants (Plaza-Diaz *et al.*, 2019; Sánchez *et al.*, 2017; Zhuang *et al.*, 2019). Approximately 200 different HMO structures have been identified, and the most abundant individual HMO is 2´-FL (Bode, 2012; Hegar *et al.*, 2019; Urashima *et al.*, 2018). The compositions of HMOs are largely determined by genetic variations in the genes that affect fucosylation, including secretor and Lewis blood group genes (Cabrera-Rubio *et al.*, 2019; Kunz *et al.*, 2017; Samuel *et al.*, 2019; Thurl *et al.*, 2010). Additionally, the concentrations of different HMOs vary over the course of lactation (Thurl *et al.*, 2017; Thurl *et al.*, 2010).

Many possible functions have been suggested for HMOs. They can function as selective prebiotics, only utilised by some bifidobacteria, such as *Bifidobacterium longum* subspecies *infantis, Bifidobacterium bifidum, Bifidobacterium breve* and *Bacteroides* spp., including *Bacteroides fragilis, Bacteroides vulgatus* and *Bacteroides thetaiotaomicron* (Chichlowski *et al.*, 2011; Marcobal *et al.*, 2010; Yu *et al.*, 2013). *In vitro* studies have shown that HMOs can prevent the adhesion of certain gut pathogens by functioning as decoy receptors (Akkerman *et al.*, 2019; Bode, 2015; Ruiz-Palacios *et al.*, 2003). These effects are both bacterial strain- and HMO structure-specific. For example, 2´-FL can reduce the adherence and colonisation of *Campylobacter jejuni* (Ruiz-Palacios *et al.*, 2003). HMOs also inhibit group B *Streptococcus* proliferation and biofilm formation, but the effects of individual HMOs vary extensively (Ackerman *et al.*, 2017a; Craft & Townsend, 2019; Lin *et al.*, 2017).

To date, most clinical studies examining individual HMOs have focused on safety and tolerance; 2´-FL and another individual HMO, lacto-N-neotetraose have been found to be safe for infants and adults (Elison *et al.*, 2016; Marriage *et al.*, 2015; Puccio *et al.*, 2017). Data regarding the effects of various HMOs on microbiota composition and activity remain scarce; however, observational studies have shown that maternal secretor status (which affects fucosylation) changes both the HMO composition (Kunz *et al.*, 2017) and the infant gut microbial composition. Therefore, fucosylation appears to increase the bifidobacterial abundance in breast-fed babies (Bai *et al.*, 2018; Lewis *et al.*, 2015; Smith-Brown *et al.*, 2016).

2.3.2.3 Galacto-oligosaccharides

GOS are established prebiotics, produced by the transglycosylation of lactose by β-galactosidases, and are composed by a mixture of galactose-based oligosaccharides with terminal glucose (Ackerman *et al.*, 2017b; Torres *et al.*, 2010). Their lengths vary from 2–10 monosaccharides, their structures may contain branching and common linkages include β (1–3), β (1–4) and β (1–6), depending on the enzyme

used and other processing conditions (Ackerman *et al.*, 2017b; Torres *et al.*, 2010). GOS are not hydrolysed by digestive enzymes but are instead fermented in the colon, primarily by bifidobacteria and lactobacilli (Davani-Davari *et al.*, 2019; Torres *et al.*, 2010).

Infants fed with GOS (or GOS:FOS at a 9:1 ratio) had larger populations of bifidobacteria and lactobacilli, and the overall composition of the microbiota was more similar to that observed for breast-fed infants than to that observed for formula-fed infants, without GOS (Akkerman *et al.*, 2019; Macfarlane *et al.*, 2008). In a piglet model, the fermentation of GOS resulted in lower colonic pH and the increased production of butyrate (Alizadeh *et al.*, 2016). *In vitro* data showed the positive effects of GOS on intestinal barrier integrity [reviewed in (Akkerman *et al.*, 2019)], and results of a piglet model, examined with and without GOS, were supportive by showing increased villus surface area and upregulated tight junction protein mRNA expression, with GOS supplementation (Alizadeh *et al.*, 2016). This study also suggested that GOS supplementation can stimulate the mucosal immune system by increasing secretory IgA in saliva (Alizadeh *et al.*, 2016). GOS consumption by elderly individuals has been shown to increase anti-inflammatory cytokines and decrease pro-inflammatory cytokines (Davani-Davari *et al.*, 2019). Both murine models and human clinical trials have indicated that dietary GOS can diminish asthma and allergic responses (Ackerman *et al.*, 2017b; Davani-Davari *et al.*, 2019).

2.4 Microbial modelling of oral cavity and colon

To address specific questions, various *in vitro* models can be useful, providing the opportunity to simplify complex conditions and representing controllable, repeatable systems that can be used to test experimental hypotheses.

Sampling from the oral cavity is quite easy and generally non-invasive. However, depending on the sampling technique used, the results can be diverse, as the oral microbiota varies significantly in different parts of the oral cavity. For caries-related biofilms, the first requirement for a micro-organism is the adherence to a surface and survival. Therefore, evaluating the properties of bacteria in biofilm models is important because the bacterial form (planktonic vs. biofilm) affects many bacterial characteristics. Ideally, a model system should feature a diverse bacterial biofilm comprised of relevant bacterial species and growth conditions that mimic those found in the oral cavity, such as a medium that resembles saliva and a surface similar to enamel (Sim *et al.*, 2016).

For the gut microbiota, *in vitro* models are even more important because sampling from the colon is highly invasive. Great interest exists in understanding how various dietary components and other compounds affect microbiota compositions and metabolism. Commonly, faecal samples are used as the inocula

for model systems. The conditions used to mimic the colon, include a pH gradient from 5–7, a temperature of 37°C and an anaerobic atmosphere (Evans *et al.*, 1988; Macfarlane *et al.*, 1998; Venema & van den Abbeele, 2013). *In vitro* models offer the means to reproducibly cultivate a complex microbial community in a controlled environment and allow for the screening of various effects associated with different compounds or bacteria, facilitating the testing of research hypotheses using models that coincide with different parts of the colon (Venema & van den Abbeele, 2013). Several *in vitro* colonic models have been presented in the literature, from static batch incubations of faecal suspensions (usually short-term) to more complex dynamic models (for which the durations vary). In this thesis, I primarily focused on dynamic models that have previously been used to evaluate changes in infant microbiota.

2.4.1 Adherence of oral microorganisms

Various types of adherence experiments are reported in the literature. Usually, the adherence of biofilm-forming bacteria in the oral cavity is tested on hydroxyapatite or other hard surfaces used to model the tooth surface. Bacteria are allowed to adhere to a surface, for a certain period of time, after which all unbound bacteria are removed, and the bound bacteria are detected. The methods used for detection include plate counting, to determine colony forming units (Lassila *et al.*, 2009), the measurement of turbidity with a spectrophotometer (Mattos-Graner *et al.*, 2000), scanning electronic microscopy (Tanner *et al.*, 2001), visualisation, using colour dyes such as crystal violet (Esberg *et al.*, 2017), fluorescent dyes, which can be detected by a plate reader (Halpin *et al.*, 2008), enzyme-linked immunosorbent assay with biotinylated bacteria (Ito *et al.*, 2017) or radioactive labels, detected by scintillation counting (Danielsson Niemi *et al.*, 2009; Esberg *et al.*, 2017; Haukioja *et al.*, 2008).

To evaluate adhesin-mediated adherence, the surface may be covered with either whole or parotid saliva, to allow pellicle formation and to mimic the conditions of the oral cavity. Alternatively, the surface may be covered with only a buffer, to decrease variability associated with saliva composition (Halpin *et al.*, 2008). Whole and parotid saliva differ in their compositions and are susceptible to individual variations (Humphrey & Williamson, 2001; Jakubovics, 2015; Jensen *et al.*, 1992). For example, *S. mutans* Ingbritt adhesion to parotid saliva-coated HA varied among 19 people, ranging from almost 20% to 70% (Wernersson *et al.*, 2006). However, the saliva-promoted adhesion of *S. mutans* also correlated well with dental plaque formation, making this a valuable tool for many experiments (Shimotoyodome *et al.*, 2007). When comparing different experiments, it is important to remember that many factors such as adherence time, cell numbers and the adhesion properties of bacterial strains vary (Van Laar *et al.*, 1996).

For bacteria that also adhere to tooth surfaces through glucans, such as *S. mutans*, alternative methods have been utilised. *S. mutans* requires sucrose for Gtfs to break the bond between glucose and fructose, allowing glucose to be utilised for the production of glucan polymers (Banas, 2004). *S. mutans* can also adhere to glucans synthesised by Gtfs on the tooth surface, via Gbps (Banas, 2004; Bowen & Koo, 2011; Koo *et al.*, 2010). Commonly, bacteria are allowed to grow and adhere in the presence of sucrose, and then, adhered bacteria are quantified, either spectrophotometrically (Hamada *et al.*, 1981; Mattos-Graner *et al.*, 2000) or using imaging techniques (Hamada *et al.*, 1981; Koo *et al.*, 2010; Söderling *et al.*, 1987).

2.4.2 Oral biofilms in batch culture

The simplest way to evaluate oral bacterial biofilms is the closed-batch culture method. A biofilm is formed on a plate wall, disc, peg, or human or animal enamel (Maciá *et al.*, 2014; Salli & Ouwehand, 2015). A batch system allows the use of either single or multiple bacterial species, including salivary or plaque bacteria, as a biofilm source and choosing suitable medium and oxygen conditions (Sim *et al.*, 2016). Often these experiments are performed in small quantities, and the length of biofilm formation and the material on which the biofilm forms can vary. One limitation is the restricted (or overly abundant) nutrient availability and the accumulation of metabolites. However, depending on the research question, batch cultures can be useful, enabling the screening of potential materials, substances or conditions, increasing the reproducibility, and is economically less demanding than other methods (Salli & Ouwehand, 2015). The most commonly used batch biofilm models are the Zürich biofilm model and the Calgary biofilm device, which have both been widely used in caries-related research [reviewed in (Salli & Ouwehand, 2015; Sim *et al.*, 2016)].

2.4.3 Continuous culture oral biofilms

More complex systems attempt to mimic more conditions in the oral cavity than batch systems. These open systems commonly utilise continuous nutrient (saliva) flow, waste removal, and pH, temperature and humidity control (Maciá *et al.*, 2014; Salli & Ouwehand, 2015). Continuous systems include chemostats, flow cells and artificial mouth models (Marsh, 1995; Salli & Ouwehand, 2015; Sim *et al.*, 2016). For a chemostat to be meaningful for caries-related research, it must include solid surfaces, to which the bacteria can adhere for biofilm formation (Bradshaw *et al.*, 1996; Sim *et al.*, 2016). Flow cells allow the formation of sequential biofilms and the opportunity for the real-time microscopic evaluation of biofilm development; however, the conditions can vary within the reactor (Salli & Ouwehand, 2015; Sim *et al.*, 2016). Artificial mouth models have been used to study microbial interactions

in a modelled biofilm and to evaluate their characteristics (Tang *et al.*, 2003). Continuous culture models usually utilise fewer replicates than batch systems; however, they offer a more controlled environment for addressing specific research questions. They are often used to evaluate bacterial consortia or a microcosm (salivary or plaque) of bacteria, but mono-species models may also be used (Salli & Ouwehand, 2015). The research question should define the appropriate experimental system. The pros and cons of the different methods have been discussed by Salli and Ouwehand (2015).

2.4.4 Dynamic colon models

The Reading simulator of the large intestine is three-stage (ascending, transverse and distal colon), continuous culture system (Gibson *et al.*, 1988; Macfarlane *et al.*, 1998). The working volume of the simulator vessels is 0.3–0.8 l, and the run time requires approximately 60 h to reach microbiological steady-state conditions (Macfarlane *et al.*, 1998). The simulator of the human intestinal microbial ecosystem (SHIME) simulates the entire gastrointestinal tract in five reactors, from the stomach to the colon (the last three reactors), with volumes ranging from 0.3–1.6 l (Molly *et al.*, 1993; Reygner *et al.*, 2016). The SHIME has an initial two-to-three-week microbiota stabilisation period before a test substrate can be added, and the retention time varies from 24 to 72 h (Pham & Mohajeri, 2018; Van de Wiele *et al.*, 2015; Van den Abbeele *et al.*, 2010; Venema & van den Abbeele, 2013). The EnteroMix colon simulator, which was used in this study, utilises smaller working volumes (6–12 ml) and models the colon in four compartments (ascending, transverse, descending and rectum) (Mäkivuokko *et al.*, 2005). The TIM-2, TNO dynamic computer-controlled *in vitro* model of the proximal colon is made from four glass jackets, connected by flexible membranes, which allows peristaltic movements to be modelled (Minekus *et al.*, 1999). The duration is shorter (1–3 days), the pH is 5.8 and the fermentation volume is approximately 0.1–0.2 l (Minekus *et al.*, 1999; Venema & van den Abbeele, 2013). TIM-2 is the only model that contains an absorption phase, simulating the intake of metabolites by the host (Minekus *et al.*, 1999; Venema & van den Abbeele, 2013). In contrast with other models, the polyfermentor intestinal model (PolyFermS) uses faecal inocula immobilised on gel beads (Zihler Berner *et al.*, 2013). PolyFermS allows the parallel testing of a control fermentation and up to four test fermentations, which are all seeded from the same inoculum reactor. However, this system utilises rather long fermentation runs, ranging from 38–79 days (Le Blay *et al.*, 2010; Pham *et al.*, 2019; Zihler Berner *et al.*, 2013). One advantage of *in vitro* colon models, in general, is the potential to mimic disturbed microbiota compositions, by adding known pathogens to the inocula (Doo *et al.*, 2017; Fehlbaum *et al.*, 2016; Forssten *et al.*, 2015). Table 1 summarises the main features of these model systems.

Table 1. The main features and differences of the common dynamic colon models [the information in the table is based on (Doo *et al.*, 2017; Dupont *et al.*, 2019; Pham & Mohajeri, 2018)].

	Reading	**SHIME**	**Enteromix**	**TIM-2**	**PolyFermS**
Volume	0.3–0.6 l	0.3–1.6 l	6–12 ml	0.1–0.2 l	0.1–0,4 l
Inoculum donor	Single donor	Single donor	Single donor	Combined or single donors	Immobilised faecal sample
Replicates	no	Up to 4	Up to 8	Up to 10	Up to 5
Inocula stabilisation time	14–17 days	14–21 days	24 hours	16 hours	5–16 days
Run time	20–96 hours –> up to17 weeks	24–72 hours –> up to 7 weeks	48 hours	1–3 days	Up to 5 weeks
Single or multi component model of colon	Multi (3-stage)	Multi (3-stage)	Multi (4-stage)	Single (proximal colon)	Single and Multi-(3-stage) versions
Advantages	Pioneering system, validated with sudden death victims. Includes mucin gels, to mimic the mucosal surface.		Many replicates. Small volumes enable the testing of scarce test products. Can be combined with cell-culture.		Immobilisation of faecal material to gel beads increases the bacterial cell density. Parallel testing option.
Limitation	No host cells. Lack of dialysis and peristalsis	Lack of peristalsis	No host cells. Lack of dialysis and peristalsis	No host cells	No host cells, lack of dialysis and peristalsis

2.4.5 *In vitro* infant colon models

The interest in understanding the effects of HMOs on the infant microbiota has increased the need to develop colonic models that focus on infants.

Previously, an infant *in vitro* colonic model has been developed in a single-culture system (Cinquin *et al.*, 2004) and a three-stage chemostat model (proximal, transverse and distal colon), using faecal bacteria immobilised on gel polysaccharide beads (Cinquin *et al.*, 2006). The experimental set-up required a stabilisation period (from 9 to 16 days), after which the fermentation was continued for 29–54 days (Cinquin *et al.*, 2004, 2006), which generated one bacterial population on gel beads and another population that was no longer adhered to the beads. In a variation of their previous system, a PolyFermS model has been used to evaluate the effects of non-protein nitrogen sources, including nucleosides and yeast extracts, on infant microbiota (from a 6-month-old, formula-fed donor) and infant microbiota when enteropathogens were included (8-month-old, formula-fed donor) (Doo *et al.*, 2017). The volumes ranged from 200 ml to 400 ml, and these fermentations lasted for 40 d and 18 d, respectively (Doo *et al.*, 2017). Increased metabolic activity and the reduced colonisation of *Salmonella* (in the infection model) were reported (Doo *et al.*, 2017). Yet another PolyFermS study evaluated the effects of lactate on the microbiota from two 2-month-old infants, for 57 days and 79 days (Pham *et al.*, 2019). These results showed that pH reductions were associated with increased lactate-producing bacteria, reduced lactate-utilising bacteria and lactate accumulation (Pham *et al.*, 2019).

An infant modification of the SHIME model (baby-SHIME) was developed, using six reactors which represent the stomach, duodenum, jejunum and ileum, ascending colon, proximal colon and distal colon, with volumes between 0.2–0.5 l, pH 3.8–6.5, and a total retention time of 28 h (De Boever, 2001). Three infants who consumed a mixed diet and solid foods (aged 3–12 months) donated faecal samples for the inoculation of colonic microbiota (De Boever, 2001).

The Copenhagen Mini Gut (CoMiniGut) model was used to evaluate the effects of 1% (w/v) 3-fucosyllactose, 3′-sialyllactose, 6′-sialyllactose and FOS on infant microbiota, representing the first report on HMOs evaluated in a colon model (Wiese *et al.*, 2018). CoMiniGut consists of five, parallel, single-vessels (each 5 ml), which are stirred under anaerobic and pH-controlled conditions, for 24 h (Wiese *et al.*, 2018). Infant faecal inocula were obtained from two vaginally born, breast-fed infants, at six months of age, and inocula were frozen until use (Wiese *et al.*, 2018). Considerable variations were observed in the simulated microbiota compositions between the two donors (Wiese *et al.*, 2018).

In another study using the Baby-SHIME model, the effect of 2g/L 2′-FL on the microbiota derived from the faecal inocula of three donors (all 6 months old, formula-fed, and started solid foods at 4 months) were used (Van den Abbeele *et al.*,

2019). This model comprises three reactors which represent the stomach and small intestine (combined in one reactor), proximal colon and distal colon with volumes and pH respectively: 140 ml, pH 3 with addition of 60 ml, pH 6; 300 ml, pH 5.4–5.6; and 500 ml, pH 6.0–6.5 (Van den Abbeele *et al.*, 2019). The system included a mucosal compartment, allowing the colonisation of the mucus layer, and required a two-week start-up period, to allow the microbiota to evolve, followed by a two-week control period and a three-week treatment period (Van den Abbeele *et al.*, 2019). 2′-FL especially increased acetate production, whereas propionate and butyrate levels were higher in the presence of 2′-FL than in controls. Differences between donors were observed in terms of lactate production (Van den Abbeele *et al.*, 2019). Only minor effects for 2′-FL were observed for the microbiota composition, although interindividual variability associated with the donors was clear (Van den Abbeele *et al.*, 2019).

3 Aims

In the present PhD thesis, we hypothesised that functional food ingredients can modulate the adhesion, colonisation, biofilm formation and virulence properties of caries-related MS and gut bacteria.

This thesis aimed to evaluate the effects of xylitol, 2′-FL and GOS on oral MS and gut microbial compositions, using developed *in vitro* methods.

The specific aims were as follows:

- To evaluate the *in vitro* effects of xylitol and sucrose, in addition to mints containing xylitol, peppermint oil and sucrose, on the early biofilm formation of *S. mutans,* using a novel dental simulator.
- To evaluate the *in vitro* effects of 2′-FL, xylitol, GOS and lactose on the growth and adhesion properties of *S. mutans.*
- To evaluate the *in vitro* effects of 2′-FL, compared with GOS and lactose, on infant colon microbial compositions and metabolites, using a novel infant colon simulation model.

4 Materials and Methods

A summary of the materials and methods is described here. More information can be found in the original publications I–IV.

4.1 Bacterial adherence and biofilm formation (I, II, III)

4.1.1 Micro-organisms and growth media

Table 2 summarises the bacterial strains used in original publications I-III: type strain *S. mutans* DSM 20523 (ATCC 25175), a reference strain *S. mutans* Ingbritt (Krasse, 1966), and *S. mutans* clinical isolates Cl 2366 and Cl 117. The origins, isolation, and identification of clinical isolates were described earlier (Söderling *et al.*, 2000; Söderling *et al.*, 2008; Söderling & Hietala-Lenkkeri, 2010). In addition, *S. sobrinus* DSM 20381 was studied (I). *S. mutans* strains and *S. sobrinus* were grown in brain heart infusion (BHI, LAB049, LabM Limited, Lancashire, United Kingdom, I and II; Becton Dickinson, Le Pont de Claix, France, III), under aerobic conditions at 37°C. For the pure culture bacterial growth experiment, tryptic soy broth (TSB, Bacto™; Becton Dickinson and Company, Sparks, MD, USA), with and without glucose, was used.

Table 2. Bacterial strains used in original publications I–III.

Bacteria	Strain	Origin	Original publication
Streptococcus mutans	DSM 20523	carious dentine	I, II, III
Streptococcus mutans	Ingbritt	dental plaque	III
Streptococcus mutans	Cl 2366	clinical isolate	I, III
Streptococcus mutans	Cl 117	clinical isolate	I
Streptococcus sobrinus	DSM 20381	dental caries	I

* DSM = Deutsche Sammlung von Mikroorganismen, Cl = clinical isolate.

4.1.2 Saliva

Paraffin-stimulated whole saliva, used in the dental biofilm simulator model (I and II), was collected previously, from 13 volunteers, pooled, filtered, pasteurised and stored at −20°C, until use (Björklund *et al.*, 2011).

Parotid saliva for the evaluation of adhesion to hydroxyapatite (III) was collected using Lashley cups, by stimulation with a Salivin lozenge (Pharmacia Ltd, Vantaa, Finland). Freshly collected parotid saliva was diluted 1:1 with buffered KCl (50 mm KCl, 0.35 mm K_2HPO4, 0.65 mm KH_2PO_4, 1.0 mm $CaCl_2$, 0.1 mm $MgCl_2$, pH 6.5) and stored on ice before experimental use.

4.1.3 Planktonic bacterial growth

The ability of three *S. mutans* strains to grow on different carbon sources was evaluated in a Bioscreen©C system (Labsystems, Helsinki, Finland) in an anaerobic cabinet (80% N_2, 10% CO_2, 10% H_2), as described earlier (Mäkeläinen *et al.*, 2010c). *S. mutans* strains were first cultured from stocks at −70°C, in BHI medium. Then, bacteria were grown overnight, in TSB with glucose, at 37°C, under aerobic conditions. For the growth experiment, 1% (v/v) bacterial suspensions were generated, in TSB devoid of glucose or any other carbon source and used immediately.

Stock solutions [10% (w/v)] of 2´-FL (DuPont Nutrition & Biosciences, Kantvik, Finland and Inbiose, Ghent, Belgium), xylitol (DuPont Nutrition & Biosciences, Kotka, Finland), glucose (J. T. Baker, Deventer, The Netherlands), lactose (Sigma-Aldrich, St. Louis, MO, USA) and GOS (kindly provided by Clasado Biosciences, St Helier, Jersey, United Kingdom) were prepared using sterile water. All stock solutions were sterile filtered (0.2 µm Minisart, Sartorius AG, Göttingen, Germany) and stored at −20°C, until use. A 20 µl volume of stock suspension for each carbon source was added to a well with 180 µl of bacterial suspension. TSB without glucose or other added carbon sources was used as a negative control. Bioscreen measured the optical density at 600 nm, every 0.5 h for 24 h. Growth curves were generated, and the area under the growth curve (AUC) for carbon sources was calculated (Mäkeläinen *et al.*, 2010c). Three independent experiments, each with triplicate samples for each carbon source, were performed.

4.1.4 Adhesion to a smooth glass surface

The exopolysaccharide mediated adhesion of *S. mutans* to a glass surface was determined, as described earlier (Mattos-Graner *et al.*, 2000). Briefly, *S. mutans* was grown in BHI, with additional 1% (w/v) sucrose (Suomen Sokeri Oy, Kirkkonummi, Finland) and 1% (w/v) of the following carbon sources: 2´-FL, xylitol, GOS, lactose and buffer, as a control. Cultures were grown in a glass tube, at a 30° angle, in an

anaerobic atmosphere at 37°C. After 18 h of growth, the unbound bacteria were transferred to another tube (planktonic bacteria). Then, the original growth tubes were rinsed with potassium-phosphate buffer (0.05 mol/l, pH 7) and lightly mixed, and the unbound bacteria were transferred to the tube containing planktonic bacteria. Potassium-phosphate buffer was added to the original growth tubes and subsequently vortexed and sonicated (Heraeus Biofuge Stratos, Kendro Laboratory Products, Langenselbold, Germany) for 30 s, to release the adhered bacteria. The tubes containing planktonic bacteria were also vortexed and sonicated. Both planktonic bacteria and adhered bacteria were quantified using a plate reader and compared against potassium-phosphate buffer (Ensight, Perkin Elmer, Waltham, MA, USA). The adhered bacteria were calculated as the ratio of adhered bacteria to all bacteria, and the results were expressed as relative adhesion, with the controls set to one. Three independent experiments, in triplicate, were performed.

4.1.5 Adhesion to saliva-coated hydroxyapatite

The adhesion to parotid saliva-coated hydroxyapatite was assessed, as described previously (Haukioja *et al.*, 2008). Briefly, *S. mutans* bacteria were first grown in BHI, overnight at 37°C. The culture was renewed with 5 µl (50 µCi) ^{35}S-labeled methionine (Perkin Elmer LifeSciences, Inc., Boston, MA, USA) and grown to the mid-logarithmic phase. Then, bacteria were washed three times with buffered KCl. Hydroxyapatite powder (Clarkson Chromatography Products Inc., South Williamsport, PA, USA) was coated with diluted parotid saliva for 1 h, under mild agitation (IKA loopster, IKA®-Werke GmbH & Co. KG, Staufen, Germany), and then hydroxyapatite was washed three times with buffered KCl. To evaluate adhesion, bacteria, parotid saliva-coated hydroxyapatite, and the tested carbon sources [1% (w/v) 2´-FL, xylitol, GOS, lactose, and plain buffer as a control] were combined, and the bacteria were allowed to adhere for 1 h, under mild agitation. Unbound bacteria were washed three times with buffered KCl, and bound bacteria were determined using a scintillation counter (MicroBeta 1450, Perkin Elmer Wallac, Waltham, MA, USA), using scintillation cocktail (Optiphase Supermix/Optiphase Hisafe 3, PerkinElmer, Waltham, MA, USA). Experiments were repeated at least twice, with 6 replicates (among which, the highest and lowest values were omitted to decrease variability). The control was set to one, and relative changes were calculated.

4.1.6 Dental biofilm simulator model

The *in vitro* dental simulator model was utilised in original publications I and II. The simulator is a bacterial biofilm model, using artificial saliva (AS) as a medium,

hydroxyapatite discs to model teeth and a continuous flow of AS to mimic the salivary flow of the oral cavity (Figs. 6 and 7). The system was briefly mentioned previously (Forssten *et al.*, 2010; Salli & Ouwehand, 2015); however, original publication I contains the first results reported using the model system.

Prior to the simulation, a fresh bacterial culture was grown to the mid-exponential phase, in BHI. Then, the culture was centrifuged, washed once with AS, and diluted to 1/4 of the original suspension. Each of the 16 simulation vessels was inoculated with 0.5 ml of the diluted bacterial suspension.

A 20% (w/v) stock suspension of the tested products was prepared in sterile water. The suspensions were then sterile-filtered (0.2 µm Minisart®, Sartorius AG). Table 3 shows the test compounds used in studies I and II. The test compounds were dissolved in AS to achieve the desired final concentration.

Table 3. Test compounds used in dental simulator studies, original publications I and II.

Test compound	Manufacturer	Original publication
sucrose	Suomen Sokeri Oy, Kantvik, Finland	I and II
xylitol	DuPont Nutrition & Biosciences, Kotka, Finland	I and II
commercial mint product with xylitol	Peppersmith Ltd, London, United Kingdom	II
commercial mint product with sucrose	Nestle UK Ltd, York, United Kingdom	II
peppermint oil	SALUS Haus Gmbh & Co KG, Bruchmühl, Germany; Emendo Oy, Vaasa, Finland, Urtegaarden Aps, Allingåbro, Denmark	II

4.1.6.1 Artificial saliva

In the dental simulator model, mucin-containing AS, mimicking bacterial growth in human saliva was used to study bacterial growth (Björklund *et al.*, 2011). The composition (salts, amino acids, vitamins, albumin, urea, myo-inositol and 5 g/l mucin) and preparation of AS were described, in detail, previously (Björklund *et al.*, 2011; Wong & Sissons, 2001).

4.1.6.2 Operation of the simulator

The *in vitro* biofilm simulator model consists of 16 bottles, in a temperature-controlled hood (B. Braun Biotech International, Sartorius AG, Göttingen,

Germany), at 37°C with continuous flow (Minipuls 3, Gilson1, Villiers le Bel, France and 202U, Watson-Marlow Ltd, Falmouth, Cornwall, England) of AS through the bottles (Figs. 6 and 7). Hydroxyapatite discs mimic teeth to offer adhesive support. Before the simulation was started, 15 ml of AS was inserted into the bottles, and the hydroxyapatite discs (Ø 7 mm, Clarkson Chromatography Products Inc.) were attached to the stainless-steel support with nail polish (Maybelline, L'Oreal, Paris, France) and covered with human whole saliva, for 1 h at 37°C, to allow pellicle formation and then inserted into the bottles. The bacterial suspension was added to the bottles and the flow was started. A constant stirring in the bottles was used to create shear forces. Samples of planktonic AS and HA discs were collected after the flow was stopped and stored at −20°C until analysed. During these experiments, the initial flow of AS was 10 ml/h, with no test products, for 0.5 h. Then, the test compounds in AS were run at 20 ml/h, for 3 h, followed by 0.5 h of incubation and a final rinsing with 10 ml/h plain AS, for 1 h. AS without the added test compounds was used as a control.

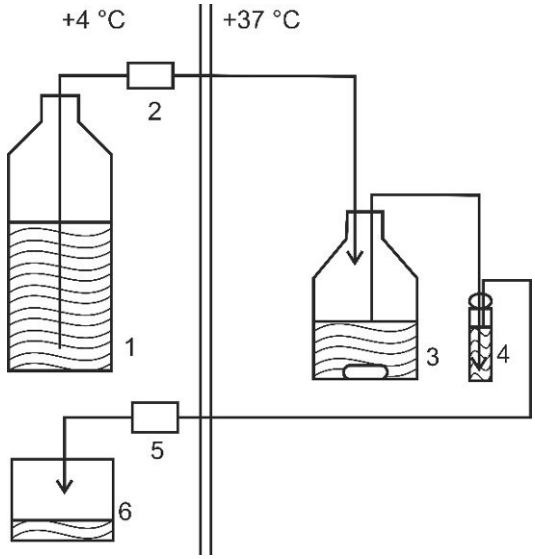

Figure 6. A schematic of the principle underlying the *in vitro* dental simulator model (reproduced from Salli and Ouwehand, 2015, under Creative Commons Attribution-Non-commercial 4.0 International License). 1. Artificial saliva container 2. Pump 3. Simulation vessel (one of 16 parallel vessels) 4. Sample collection during simulation 5. Outlet pump 6. Waste.

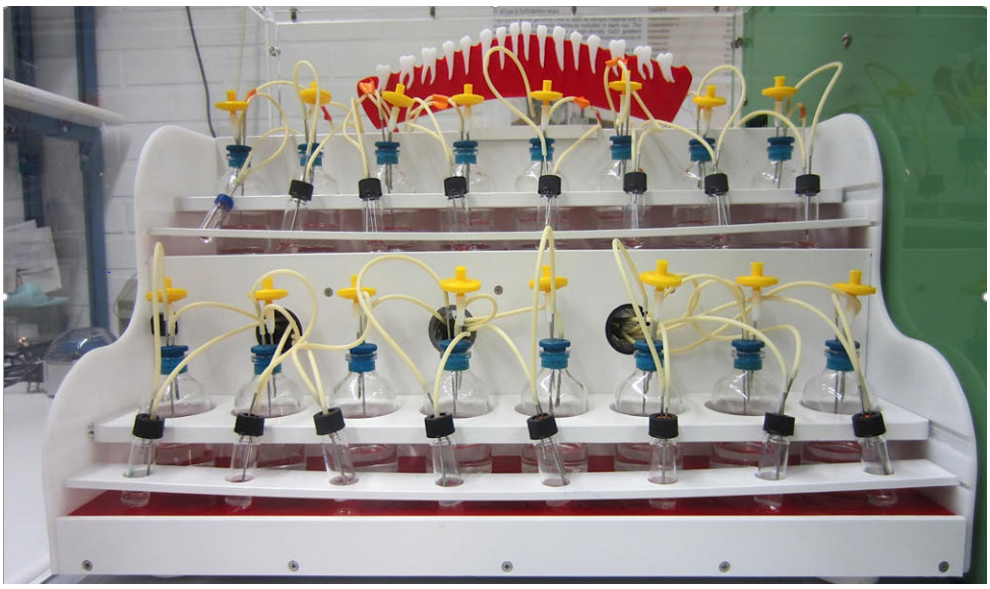

Figure 7. Photo of the dental simulator vessels (copyright Krista Salli).

4.1.6.3 DNA extraction

DNA was extracted from bacteria that adhered to hydroxyapatite discs, as described by Wilson, with minor modifications (Wilson, 2001). First, the hydroxyapatite discs were rinsed to remove the loosely adhered bacteria. Hydroxyapatite discs were then placed in a solution of 12% sucrose, in 25 mM Tris-HCl (pH 8.0), lysozyme was added and the discs were incubated for 2 h, at 37°C. Then, 10% SDS, 250 mM EDTA (pH 8.0) and proteinase K were added, and the mixture was incubated for 2 h at 37°C, followed by the addition of NaCl, mixing, the addition of cetyl trimethyl ammonium bromide, and incubation for 20 min at 65°C. Finally, DNA was extracted using a chloroform:isoamyl alcohol (24:1) mixture and precipitated using isopropanol. DNA was collected by centrifugation, washed with 70% ethanol, resuspended in elution buffer (Ambion Inc., Austin, TX, USA) and stored at −20°C.

DNA from planktonic AS samples was extracted using the MagMAX™Total Nucleic Acid Isolation Kit (Ambion Inc.), according to the manufacturer's instructions, using the MagMAX™ Express 96 sample preparation system (Life Technologies, Halle, Belgium). Bead beating was performed with Precellys24 (Bertin Technology, Montigny le Bretonneux, France), and DNA concentration was measured using a Nanodrop ND-1000 Full-spectrum UV/Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

4.1.6.4 qPCR

In original publication I, *S. mutans* and *S. sobrinus* from the planktonic AS samples and HA discs were quantified using quantitative polymerase chain reaction (qPCR) with Applied Biosystems Real-Time PCR equipment (ABI 7500 FAST, Applied Biosystems, Foster City, CA, United States) and software. An *S. mutans*-specific assay utilised TaqMan Master Mix, without AmpErase UNG (Applied Biosystems), 300 nmol of forward and reverse primers and 200 nmol of the probe. To detect *S. sobrinus* DSM 20381, a *Streptococcu*s-specific assay was utilised with Power SYBR Green Master Mix, without AmpErase UNG (Applied Biosystems, Bridgewater, NJ, USA) and 300 nmol of each primer. The reaction volume used in both assays was 25 ml, and 1 ng of template DNA was used. The primers used in this study are shown in Table 4. In original publication II, *S. mutans* was determined using a *Streptococcus* assay.

The amplification profile for both assays was as follows: 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. A 10-fold dilution series, from 1 pg to 1 ng, of the target species *S. mutans* type strain was included to create a standard curve. Water was used as a no template control, and assays in which the no template controls had Cq values 5–10 cycles below that of the lowest sample were accepted. Triplicate samples were analysed, and the results were expressed as $\log_{10}$ genomes per ml AS or per hydroxyapatite disc, considering the size and the 16S rDNA copy number of the standard species genome.

Table 4. Primers and probe used in the qPCR analysis

Designation	Sequence	Target gene	Reference	Original publication
Smut_fwd primer	50-GTCTACAGCTCAGAGATGCTATTCT-30	*gtf*B	Modified from a	I
Smut_rev primer	50-GCCATACACCACTCATGAATTGATAAT-30		Modified from a	I
Smut_fwd probe	50-FAM-TGGAAATGACGGTCGCCGTTATGAA-BHQ1-30		Modified from a	I
Str1 primer	50-GTACAGTTGCTTCAGGACGTATC-30	*tuf*	b	I, II
Str2 primer	50-ACGTTCGATTTCATCACGTTG-30		b	I, II

* a is (Yoshida *et al.*, 2003); b is (Picard *et al.*, 2004).

4.1.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software, La Jolla, CA, USA), version 6.04 (original publications I and II) and version 8.1 (original publication III). P-values less than or equal to 0.05 were considered significant. In the dental simulator studies (I and II) the differences between treatment groups were analysed by one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. In original publication I, the effects of increasing concentrations of xylitol combined with 1% sucrose were tested using nonlinear regression, semi logline analysis. In original publication II, comparisons of the ratios of HA-attached to planktonic bacteria were performed using two-sided, non-paired, student's t-test (Excel in Microsoft Office 365 ProPlus). The results were analysed from at least three (Study I) or two (Study II) independent experiments. Original publications I and II examined each treatment in duplicate for each experiment. In original publication III, significant differences between groups were analysed using one-way ANOVA and Dunnett's multiple comparisons test, for the growth experiment, and Tukey's multiple comparisons test, for the adhesion experiments.

4.2 EnteroMix colon model (IV)

The semi-continuous EnteroMix colon simulator model was first described by Mäkivuokko *et al.* (2005, 2006). This model was developed based on the Reading three-stage model (Gibson *et al.*, 1988; Macfarlane *et al.*, 1998) and has previously been used to study the effects of pre- and probiotics on the microbiota composition and metabolites of microbial inocula obtained from adult volunteers (Mäkeläinen *et al.*, 2010a; Mäkivuokko *et al.*, 2010; van Zanten *et al.*, 2012). The original publication IV was the first to investigate the effects of HMOs on the infant microbiota composition and metabolites with EnteroMix model. The present study was reviewed and approved by the Coordinating Ethical Committee of the University of Helsinki (Decision number 139/13/03/00/16). All methods were performed in accordance with the national guidelines of Finland. One simulator unit is composed of four parallel vessels, V1 to V4 (Figs. 8 and 9). The conditions of the vessels were adjusted to mimic the different parts of the colon: ascending (V1), transverse (V2), descending (V3) and the end of the descending/rectum area (V4). The pH increased as the system moved from V1-V4 (5.5–7.0) and was adjusted using gaseous ammonia if it fell below the set target value. The volumes increased from V1–V4 (6 ml–12 ml), to model reduced flow. The temperature of the simulator was maintained at 37°C, in an anaerobic atmosphere, and customised software was used to control the pH and the gas and liquid transfers during the simulation.

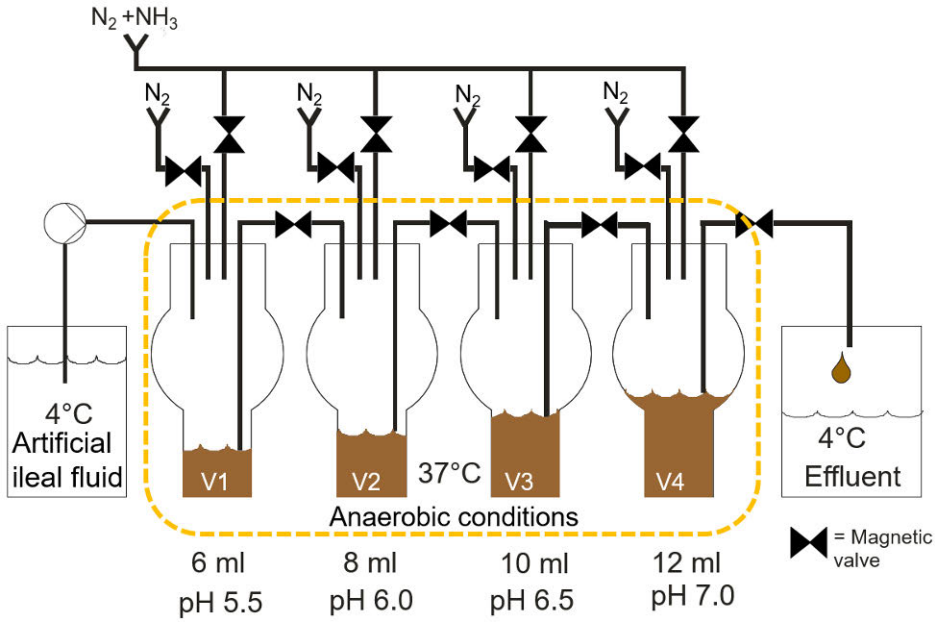

Figure 8. A schematic showing a single unit of the EnteroMix colon simulator system. Vessel 1 (V1, proximal) to vessel 4 (V4, distal) represent different parts of the colon. Nitrogen was used to maintain anaerobiosis and as a carrier gas for ammonia and liquid transfers. The system was computer-controlled [own drawing, modified from (Mäkivuokko *et al.,* 2006)].

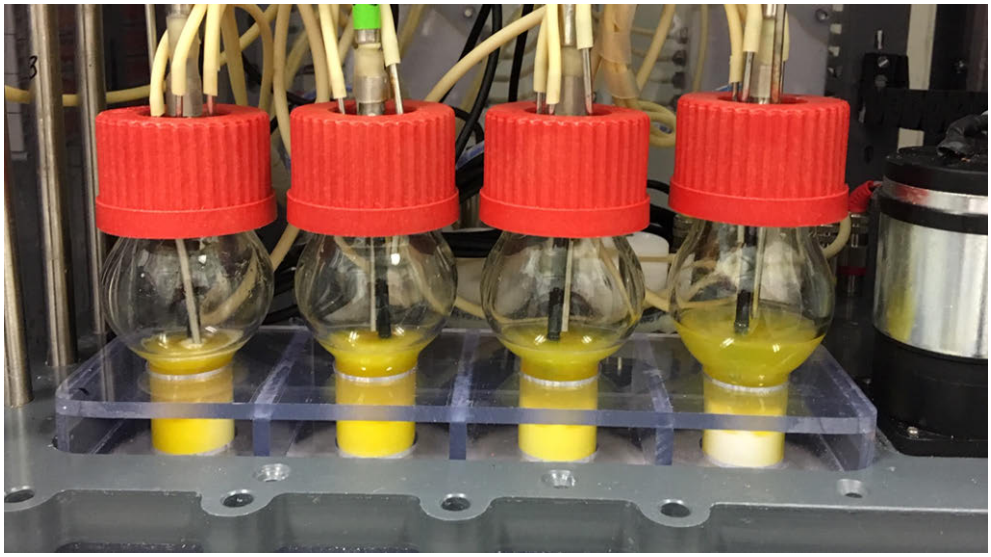

Figure 9. A picture of one unit of the colon simulator, with vessels representing the proximal to distal parts of the colon (copyright by Krista Salli).

4.2.1 Artificial ileal fluid

A version of synthetic ileal fluid is often used as a medium in colon simulator runs. As described previously (Gibson *et al.*, 1988), a slightly modified version of artificial ileal fluid was used in these colon simulations (Macfarlane *et al.*, 1998; Mäkivuokko *et al.*, 2005). The components were dissolved in water in the following amounts (g/l): starch, 5.0; peptone, 0.05; tryptone, 5.0; yeast extract, 5.0; NaCl, 4.5; KCl, 4.5; mucin (porcine, type II), 4.0; casein, 3.0; pectin, 2.0; xylan (oat spelt), 2.0; arabinogalactan, 2.0; $NaHCO_3$, 1.5; $MgSO_4$, 1.25; guar gum, 1.0; inulin, 1.0; cysteine, 0.8; KH_2PO_4, 0.5; K_2HPO_4, 0.5; bile extract (porcine), 0.4; $CaCl_2 \times 6\ H_2O$, 0.15; $FeSO_4 \times 7\ H_2O$, 0.005; hemin, 0.05 and Tween 80, 1.0. Before being used in the simulator, the medium was prepared by mixing 1 part of the above described, anaerobic, autoclaved fluid with 2 parts of pre-reduced 20 mM NaH_2PO_4, pH 6.5 and 1 part of pre-reduced 0.9% NaCl.

4.2.2 Tested products

To evaluate the effects of 2′-FL (DuPont Nutrition & Biosciences and Inbiose), lactose (Sigma-Aldrich) and GOS (Clasado Biosciences), 2% (w/v) solutions were prepared in artificial ileal fluid. Artificial ileal fluid, without added carbohydrates, was used in a control simulation.

4.2.3 Inocula donated by infants

Infant faecal samples were used to inoculate the colon simulator system. Infants who donated faecal samples were between 0.5 and 8 months old, in good health, and had not been medicated with antibiotics. A parent for each infant provided informed consent and background information, including the age, food, supplements, allergies and delivery mode of the infant. Parents were provided with materials for sample collection and handling. They were instructed to freeze the faecal samples, immediately, at −20°C, before the samples were transported to the laboratory and stored at −80°C. Faeces from a single infant donor was used to inoculate a set of four parallel simulations. To obtain sufficient faeces from the same infant, we used frozen faeces and pooled all samples from a single infant that were collected within one week. The samples from a single donor were mixed with three parts artificial ileal fluid, filtered through a 0.3-mm metal mesh, and incubated anaerobically, for 24 h at 37°C, before being added to the simulator.

4.2.4 Operation of the simulator

Before the simulation began, the simulator system was first flushed with nitrogen gas, and vessels V1, V2, V3 and V4 were filled with water and an anaerobic

physiological salt solution containing resazurin, to volumes of 3, 5, 7 and 9 ml, respectively. Then all units were inoculated with faecal samples in V1. The faecal inoculum was then transferred to V2, from there to V3, and then to V4, before being deposited in the waste container. When a simulation run was started, the media was transferred at three-hour intervals by a pressurised gas, in the following order: V4 to effluent, V3 to V4, V2 to V3, V1 to V2, and 3 ml of new artificial ileal fluid, with and without test products (control), to V1. After 48 h, the simulation was stopped, and samples were removed from each vessel. The final volumes in vessels V1, V2, V3 and V4 were 6, 8, 10 and 12 ml, respectively.

4.2.5 Sample analysis

Samples were taken from each vessel at the end of the 48-h simulation and maintained at –20°C until analysis.

4.2.5.1 2´-FL and fucose concentration

The 2´-FL and fucose concentrations (from the simulation units to which 2´-FL was added) were determined, as described in original publication IV. Briefly, standard fucose (Sigma-Aldrich, St. Louis, MO, USA) and 2´-FL (DuPont Nutrition and Biosciences) solutions with known concentrations were made. Samples were centrifuged, mixed with ethanol, incubated and re-centrifuged, and the supernatant was evaporated to dryness, dissolved in water and filtered. High-performance anion-exchange chromatography, with a mobile phase consisting of water and NaOH gradient, was applied. The retention times for fucose and 2´-FL were 6.3 min and 22.8 min, respectively.

4.2.5.2 Analysis of microbial numbers

The total bacterial numbers were analysed using flow cytometry, as described previously (Apajalahti *et al.*, 2002). Briefly, the samples for flow cytometry analysis were fixed with 4% formaldehyde when the simulation was stopped, and the samples were stored at 4°C until analysis. Bacteria were stained with SYTO24 dye (Molecular Probes, Leiden, The Netherlands) and counted with the FACSCalibur system (BD Biosciences, San Jose, CA, USA).

DNA was extracted using the MagMAX™ with Total Nucleic Acid Isolation Kit (Ambion Inc.) and the Mag MAX™ Express 96 sample preparation system (Life Technologies), according to the manufacturer's instructions. A Precellys24 homogeniser (Bertin Technology) was used for bead beating, and DNA was further purified with the One-Step-96^{TM} PCR Inhibitor Removal Kit (Zymo Research,

Irvine, CA, USA). DNA concentrations were measured using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Total bifidobacteria were quantified by real-time qPCR, using TaqMan and Applied Biosystems Real-Time PCR equipment and software (ABI 7500 FAST, Applied Biosystems), as described previously (Mäkeläinen *et al.*, 2010a; Mäkivuokko *et al.*, 2005). The amplification assays were performed as follows: activation at 95°C for 20 s, and 40 cycles of denaturation at 95°C for 3 s and annealing at 60°C for 30 s. Standard curves, consisting of 10-fold dilutions of target species DNA, were used for quantification.

4.2.5.3 Microbial composition by sequencing

DNA extraction and purification were described in the previous section. The 16S rRNA gene, at the V4 variable region, was amplified from all simulation samples, as described previously (Caporaso *et al.*, 2012; Raza *et al.*, 2017). The amplicon pool was sequenced using the Illumina MiSeq system, with 2 × 250 bp reads (DuPont Pioneer, Johnston, IA, USA), and examined using the Quantitative Insights Into Microbial Ecology pipeline (QIIME v. 1.9.1) (Caporaso *et al.*, 2010; Caporaso *et al.*, 2012; Raza *et al.*, 2017). Sequences were clustered into operational taxonomic units, at 97% sequence similarity, against the Greengenes database (v. 13.8) (DeSantis *et al.*, 2006). Taxa compositions were reported as relative abundance (% of total sequences) and visualised using Microsoft Excel.

4.2.5.4 The concentrations of SCFAs and BCFAs

The concentrations of SCFAs and BCFAs from the simulation samples were analysed, as described previously, using gas chromatography (Ouwehand *et al.*, 2009). In brief, pivalic acid, as an internal standard, was added to each sample. After mixing, centrifugation, the addition of oxalic acid, incubation, and re-centrifugation, the supernatant was analysed using a glass column, packed with 80/120 Carbopack B-DA/4% on Carbowax 20M stationary phase (Supelco, Bellefonte, PA, USA), at 175°C, using helium as the carrier gas and a flame ionisation detector. The concentrations of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, 2-methylbutyric acid and lactic acid were determined.

4.2.5.5 The concentrations of biogenic amines

Biogenic amines were determined, as described by Saarinen, using high performance liquid chromatography (Saarinen, 2002). Methylamine, ethylamine, tryptamine, β-phenyl-ethylamine, 2-methyl-butylamine, putrescine, cadaverine, histamine,

tyramine, spermidine and spermine were analysed. In brief, the samples were prepared by the addition of heptylamine, as an internal standard, and then all amines were extracted, and the proteins were precipitated with perchloric acid. The derivatisation of the samples and standards was performed by first adding NaOH, saturated sodium bicarbonate solution, and a derivatisation reagent (1% dansyl chloride in acetone). Then, the samples were mixed, incubated, and ammonia was added, followed by mixing, incubation, the addition of acetonitrile, mixing, and centrifugation. The separation was performed under reversed-phase conditions, with a Spherisorb ODS-2 column (Waters, Helsinki, Finland), using a gradient elution of 0.02 M ammonium acetate and acetonitrile, and detected with an ultraviolet detector.

4.2.6 Statistical analysis

The statistical analysis was explained, in detail, in original publication IV. Differences in microbial composition, based on the relative abundance of taxa (> 0.1% abundance), were determined using the Kruskal-Wallis test, and p-values were adjusted by the Benjamini-Hochberg false-discovery rate (FDR) correction (Benjamini & Hochberg, 1995). Taxa with FDR corrected p-values <0.05 were considered significantly different.

Data for all microbial metabolites were analysed as longitudinal data, across multiple vessels, using the nonparametric and robust methods that were developed by Brunner and colleagues (Brunner *et al.*, 2002), which are part of the R package, *nparLD* (Noguchi *et al.*, 2012). P-values from multiple, simultaneous tests were Benjamini-Hochberg FDR-corrected (Benjamini & Hochberg, 1995), and p-values of 0.05 or less were considered significant.

Data regarding the numbers of bifidobacteria and total bacteria were analysed, using a linear, mixed-effects model, with random intercepts and slopes for the subjects, and using vessel number as a continuous covariate. Another fixed, second-order slope was added to account for the nonlinear growth of bacteria in the vessels. Statistically non-significant interaction terms were excluded, to obtain increased power for the estimation of the parameters of interest.

5 Results

The results of original publications I-IV are presented here.

5.1 Effects of 2´-FL, GOS and xylitol on the planktonic growth of *Streptococcus mutans* (III)

We evaluated the ability of three *S. mutans* strains, DSM 20523, Ingbritt and Cl 2366, to grow on different carbon sources. The growth medium, without any added carbon source, was used as a negative control (TSB– + inocula), whereas medium with glucose added was used as a positive control. Optical density (OD) at 600 nm was measured, using Bioscreen equipment under anaerobic conditions, every 30 min for 24 h. All tested *S. mutans* strains grew well with 1% (w/v) glucose, lactose and GOS; however, they did not grow with 1% (w/v) 2´-FL as their carbon sources. Xylitol, at 1% (w/v), significantly inhibited the growth of the studied *S. mutans* strains, compared with the control with no added carbon sources (Fig. 10).

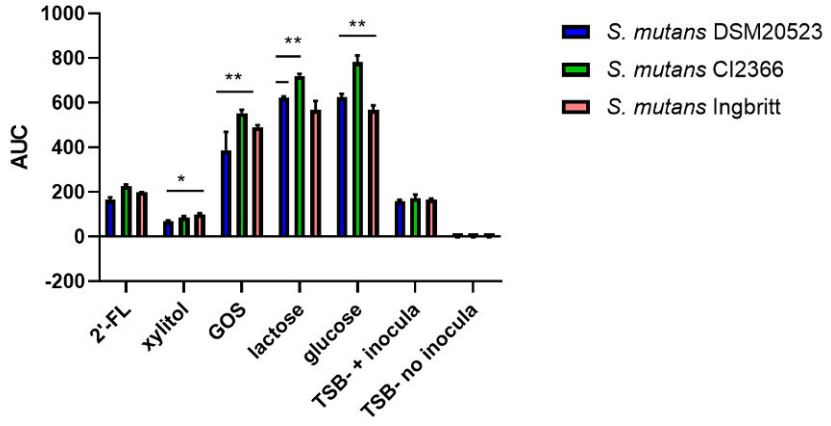

Figure 10. The growth of *S. mutans* DSM 20523, Cl 2366 and Ingbritt, using different carbon sources. * $p < 0.05$; ** $p < 0.001$ in comparison to TSB– + inocula. TSB– = tryptic soy broth without glucose; 2´-FL = 2´-fucosyllactose; GOS = galacto-oligosaccharides; AUC = area under the growth curve; TSB = tryptic soy broth. This represents a modification of the Figure 1 in original publication III.

5.2 Effects of 2´-FL and GOS on *Streptococcus mutans* adherence to a smooth glass surface (III)

We evaluated the exopolysaccharide-mediated adhesion of the *S. mutans* strains DSM 20523, Ingbritt and Cl 2366 to a glass surface, in the presence of 1% (w/v) 2´-FL, xylitol, GOS and lactose. No increases in *S. mutans* adhesion were observed for any of the studied test compounds. For *S. mutans* Ingbritt, no significant changes were found with any of the studied compounds. The relative adhesion of type strain DSM 20523 was significantly ($p < 0.05$) reduced by 2´-FL, GOS, and lactose, compared with the control, whereas the strain CI 2366 only showed a reduction with GOS.

5.3 Effects of 2´-FL, GOS and xylitol on *Streptococcus mutans* adherence to saliva-coated hydroxyapatite (III)

We evaluated the adhesin-mediated adhesion to a parotid saliva-coated hydroxyapatite surface for the *S. mutans* strains DSM 20523, Ingbritt and Cl 2366, in the presence of 1% (w/v) 2´-FL, xylitol, GOS and lactose. Compared with the control, no changes in adhesion were found with any of the studied compounds for the strains DSM 20523 and Ingbritt. In contrast, 2´-FL and GOS decreased the adhesin-mediated adhesion to parotid saliva-coated HA for the strain CI 2366 ($p < 0.05$).

5.4 Effects of xylitol concentrations on the biofilm formation of *Streptococcus mutans* (I)

We studied the effects of increasing 2%–5% (w/v) xylitol concentrations, with and without 1% (w/v) sucrose, using the *S. mutans* type strain DSM 20523. The results for both hydroxyapatite-adhered and planktonic AS bacteria are presented in Table 5. The addition of sucrose to AS significantly increased both the adhered and planktonic bacterial numbers. In contrast, the addition of xylitol to AS, at concentrations ranging from 2% to 5%, significantly decreased the amount of *S. mutans* that adhered to hydroxyapatite, compared with AS. For planktonic bacteria, significant decreases were found at xylitol concentrations of 2% and 4%. The addition of xylitol (2% to 5%) to 1% sucrose in AS resulted in a decrease in bacterial numbers adhered to hydroxyapatite with increasing xylitol concentration in comparison to AS with 1% sucrose. In AS samples, the addition of xylitol to sucrose-AS, showed no changes.

Table 5. Bacterial numbers for *S. mutans* DSM 20523 (mean ± std) biofilm adhered to hydroxyapatite (HA) discs and from planktonic artificial saliva (AS) in a dental simulator. DNA was extracted and quantified using real-time qPCR. Significance was determined by ANOVA and Tukey's multiple comparisons test. P- values: $p < 0.05$ (a) in comparison to AS alone, and (b) in comparison to AS with 1% sucrose.

Test compound added to AS	Log_{10} *S. mutans*/HA disc	Sample n	Signi-ficance	Log_{10} *S. mutans*/ml AS	Sample n	Signi-ficance
AS alone	4.5 ± 0.4	19	-	6.9 ± 0.4	20	-
1% sucrose	5.9 ± 0.3	20	a	7.4 ± 0.3	20	a,b
2% xylitol	3.6 ± 0.3	10	a, b	5.9 ± 0.4	10	a
2% xylitol + 1% sucrose	5.8 ± 0.4	10	a	7.1 ± 0.5	10	
3% xylitol	3.7 ± 0.7	11	a, b	6.5 ± 0.4	11	b
3% xylitol + 1% sucrose	5.6 ± 0.4	10	a	7.2 ± 0.5	10	
4% xylitol	3.0 ± 0.5	6	a, b	6.2 ± 0.4	5	a,b
4% xylitol + 1% sucrose	5.2 ± 0.4	6	a, b	7.0 ± 0.2	6	
5% xylitol	3.5 ± 0.3	6	a, b	6.4 ± 0.2	6	b
5% xylitol + 1% sucrose	5.2 ± 0.7	6	a	7.0 ± 0.4	6	

5.5 Effect of xylitol and sucrose mints on biofilm formation of *Streptococcus mutans* (II)

We evaluated the effects of commercial xylitol mints and sucrose mints on the early biofilm formation of *S. mutans* DSM 20523 (Fig. 11). The mint pastilles also contained peppermint oil (PO); therefore, PO, xylitol and sucrose were also evaluated separately. Both 3% sucrose and 3% sucrose mints significantly increased the numbers of *S. mutans* in the hydroxyapatite adhered biofilm, whereas 3% xylitol and 3% xylitol with PO decreased the numbers, compared with the AS control. For 3% xylitol mints, however, the decrease did not reach significance. The combination of xylitol with PO resulted in significantly reduced biofilm bacterial numbers compared with PO alone.

Sucrose increased the planktonic bacterial *S. mutans* numbers, whereas xylitol, xylitol mints, and xylitol with PO decreased the numbers. In addition, both xylitol and xylitol with PO decreased *S. mutans* numbers in comparison with PO alone.

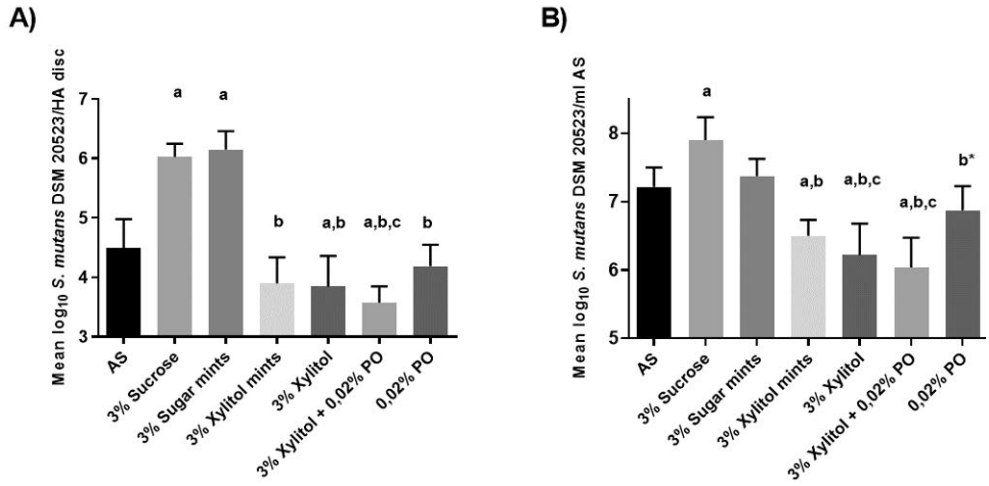

Figure 11. The effects of 3% (w/v) sucrose, sugar mints, xylitol mints, xylitol, xylitol with 0.02% peppermint oil (PO) and 0.02% PO, in artificial saliva (AS), on the numbers (mean ± std) of hydroxyapatite (HA)-attached biofilm and planktonic *S. mutans* DSM 20523, in a dental simulator. DNA was extracted from A) HA discs and B) planktonic AS, and bacteria were quantified by real-time qPCR. Significance ($P < 0.05$) is indicated by (a) compared with AS control, (b) compared with sucrose and sugar mints (b* is only significant in comparison to sucrose) and (c) compared with PO (ANOVA and Tukey's multiple comparisons test). This represents a modification of the Figures 1 and 2 in original publication II.

5.6 Effects of xylitol and sucrose on the biofilm formation of mutans streptococci (I)

We studied the ability of the *S. mutans* strains DSM 20523, Cl 2366 and Cl 117 and the *S. sobrinus* strain DSM 20381 to form early biofilms on hydroxyapatite in the presence of 2% (w/v) xylitol, 1% (w/v) sucrose and 2% (w/v) xylitol combined with 1% (w/v) sucrose. The numbers of hydroxyapatite adhered and planktonic AS salivary bacteria are presented in Table 6. Sucrose increased the biofilm formation of all tested bacteria compared with the AS control. Xylitol (2%) decreased biofilm formation for all strains, except the clinical isolate Cl 117, compared with the AS control. Xylitol combined with sucrose decreased the biofilm formation of clinical isolate Cl 2366 compared with sucrose alone. The effects observed for planktonic bacteria were smaller. Sucrose increased planktonic bacterial numbers only for *S. mutans* DSM 20523 but not for *S. sobrinus* DSM 20381 or the two clinical isolates. Xylitol decreased the bacterial numbers of *S. mutans* DSM 20523, CI 2366 and *S. sobrinus* DSM 20381, whereas xylitol increased the numbers of CI 117.

Table 6. Bacterial numbers (mean ± std) for 1) *S. mutans* DSM 20523, 2) *S. mutans* CI 2366, 3) *S. mutans* CI 117 and 4) *S. sobrinus* DSM 20381 adhered to hydroxyapatite (HA) discs and in planktonic artificial saliva (AS) samples, in a dental simulator. DNA was extracted and quantified using real-time quantitative polymerase chain reaction. Significance was determined by ANOVA and Tukey's multiple comparisons test. P- values: $p < 0.05$ (a) in comparison to AS alone, and (b) in comparison to AS with 1% sucrose.

1) DSM 20523 Test compound added to AS	**Log_{10} *S. mutans*/HA disc**	**Sample n**	**Signi-ficance**	**Log_{10} *S. mutans*/ ml AS**	**Sample n**	**Signi-ficance**
AS alone	4.3 ± 0.3	8	-	6.6 ± 0.4	8	-
1% sucrose	6.0 ± 0.2	8	a	7.4 ± 0.4	8	a,
2% xylitol	3.6 ± 0.3	8	a, b	5.9 ± 0.3	8	a,b
2% xylitol + 1% sucrose	5.8 ± 0.4	8	a	7.1 ± 0.6	8	

2) CI 2366 Test compound added to AS	**Log_{10} *S. mutans*/HA disc**	**Sample n**	**Signi-ficance**	**Log_{10} *S. mutans*/ml AS**	**Sample n**	**Signi-ficance**
AS alone	4.5 ± 0.4	10	-	6.5 ± 0.4	10	-
1% sucrose	5.7 ± 0.6	10	a	6.6 ± 0.6	9	
2% xylitol	3.6 ± 0.4	10	a, b	5.5 ± 0.4	10	a,b
2% xylitol + 1% sucrose	5.0 ± 0.7	10	b	5.8 ± 0.5	10	a,b

3) CI 117 Test compound added to AS	**Log_{10} *S. mutans*/HA disc**	**Sample n**	**Signi-ficance**	**Log_{10} *S. mutans*/ ml AS**	**Sample n**	**Signi-ficance**
AS alone	3.7 ± 0.2	8	-	5.3 ± 0.4	8	-
1% sucrose	4.9 ± 0.3	8	a	5.7 ± 0.3	8	
2% xylitol	3.9 ± 0.3	8	b	6.0 ± 0.3	8	a
2% xylitol + 1% sucrose	5.1 ± 0.6	8	a	6.0 ± 0.4	8	a

4) DSM 20381 Test compound added to AS	**Log_{10} *S. mutans*/HA disc**	**Sample n**	**Signi-ficance**	**Log_{10} 0 *S. mutans*/ml AS**	**Sample n**	**Signi-ficance**
AS alone	3.6 ± 0.4	8	-	6.6 ± 0.3	8	-
1% sucrose	5.2 ± 0.4	8	a	6.7 ± 0.2	8	
2% xylitol	3.0 ± 0.4	8	a, b	5.9 ± 0.4	8	a,b
2% xylitol + 1% sucrose	5.2 ± 0.5	8	a	6.8 ± 0.4	8	

5.7 Effects of 2′-FL and GOS on simulated infant colonic microbiota and metabolites (IV)

Infant colon simulations were performed, to evaluate the effects of 2% (w/v) 2′-FL, GOS, lactose and control (no added carbohydrates in the synthetic ileal fluid) on the *in vitro* simulated, infant microbiota composition and microbial metabolites. The results from vessels V1-V4 were combined in some analyses, to improve the comprehensibility of the results. The results were evaluated by combining the results from all nine simulations combined. The nine simulations were then divided into breast-fed (BF) and formula-fed (FF) groups, based on the information provided for the faecal sample inocula donors. In addition, the simulations were grouped according to the utilisation of 2′-FL by microbiota, into 2′-FL fast-fermenting and slow-fermenting groups. In this thesis, I focused primarily on the effects of 2′-FL, GOS and lactose, compared against the no-added-carbohydrate control.

5.7.1 Infants who donated faecal inocula

Nine *in vitro* colon simulations were performed. The infants who donated the faecal samples for inocula ranged from 2 weeks to 8 months old. The group of BF donor infants (n = 5) included one infant who was rarely fed with formula, whereas the others were exclusively BF. The three FF donors used commercially available formula as their primary source of food. One FF infant donated two samples (008 and 013), at different time points (5.5 and 7.5 months). One BF donor and two FF donors had started the use of solid foods at the time of sample collection. Only one infant was delivered by caesarean section, and all but one used probiotic supplementation. The total bacterial numbers were high in all inocula with a mean population of $\log_{10}$ 9.6 ± 0.3 cells/ml (mean ± std, n = 9). In addition, total bifidobacteria, as analysed by qPCR, was relatively high, at $\log_{10}$ 9.6 ± 0.4 cells/ml (mean ± std, n = 7); however, for two infants (002, BF and 012, FF), bifidobacterial levels were below the detection limit.

5.7.2 2′-fucosyllactose and fucose levels

2′-FL and fucose utilisation were analysed from the simulator units to which they were added. Fig. 12 shows the 2′-FL levels, which were used to group the simulations according to the 2′-FL fermentation capability, into 2′-FL fast-fermenting (004, 008 and 011, circled in Fig. 12) and 2′-FL slow-fermenting groups (n = 6), to better compare the differences in the microbiota compositions and metabolites. Fucose is a secondary metabolite of 2′-FL utilisation. The results of the fucose levels were reported in Study IV.

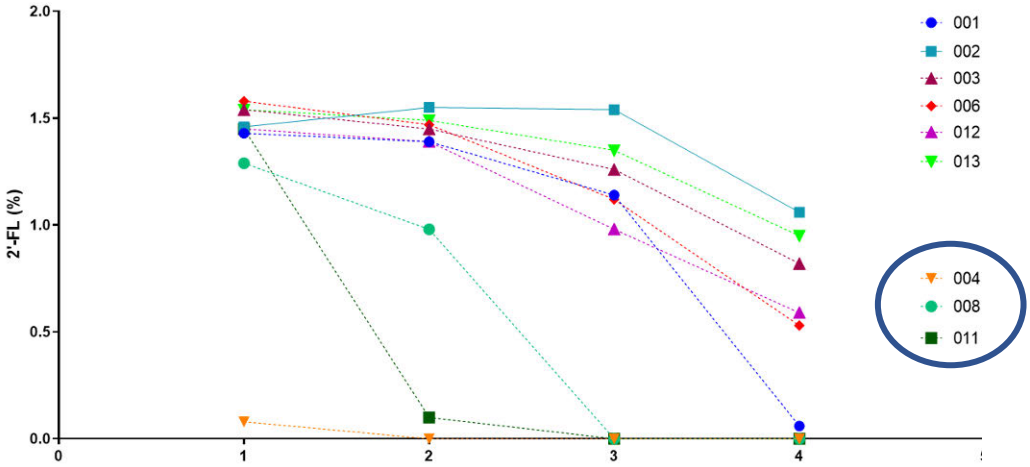

Figure 12. The concentrations of 2′-fucosyllactose (2′-FL) from each simulation in each simulation vessel.

5.7.3 Total bacterial and total bifidobacterial numbers

We determined the total bacterial numbers by flow cytometry and the total bifidobacterial numbers by qPCR, from the simulation samples. Total bacterial numbers increased from V1–V4 in all simulations ($p < 0.001$), as did bifidobacterial numbers ($p < 0.001$), regardless of the groupings. However, the 2′-FL fast-fermenting group had larger bacterial numbers than the 2′-FL slow-fermenting simulations ($p < 0.001$).

2′-FL, GOS and lactose simulations resulted in higher bifidobacterial numbers (2′-FL, $p = 0.023$; lactose, $p = 0.009$ and GOS, $p = 0.005$) compared with the control simulations, when all simulations were combined. The grouping of simulations into BF and FF or 2′-FL fast- and slow-fermenting groups was also evaluated.

5.7.4 Microbiota composition

The microbiota composition was determined by 16S rRNA sequencing performed for each vessel. To simplify the visualisation of the results, the results from V1-V4 were pooled. Fig. 13 a–c shows the effects of treatments with 2′-FL, lactose and GOS, compared with the control, in addition to comparisons according to both the feeding mode of the inocula donors (BF vs. FF) and the 2′-FL fermentation rate (slow vs. fast), on the relative abundance of bacteria at the phylum level. 2′-FL, GOS and lactose increased the abundance of Firmicutes compared with the control, and Proteobacteria abundance decreased in both lactose and GOS simulations. Verrucomicrobia was more abundant in FF compared with BF infants. Actinobacteria increased in the 2′-FL fast-fermenting simulations,

whereas more Proteobacteria was observed in the 2´-FL slow-fermenting simulations.

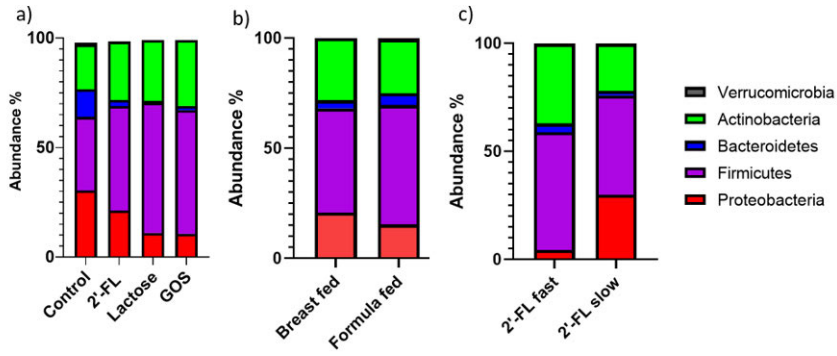

Figure 13. The microbiota compositions at the phylum level. a) The effects of 2´-fucosyllactose (2´-FL), lactose and galacto-oligosaccharides (GOS), compared with the control. b) Simulations were grouped according to the inocula donor feeding type, as either breast-fed or formula-fed. c) Simulations were grouped according to the 2´-FL fermentation type, into either 2´-FL fast or slow-fermenting groups. All vessels and all simulations were combined for this analysis.

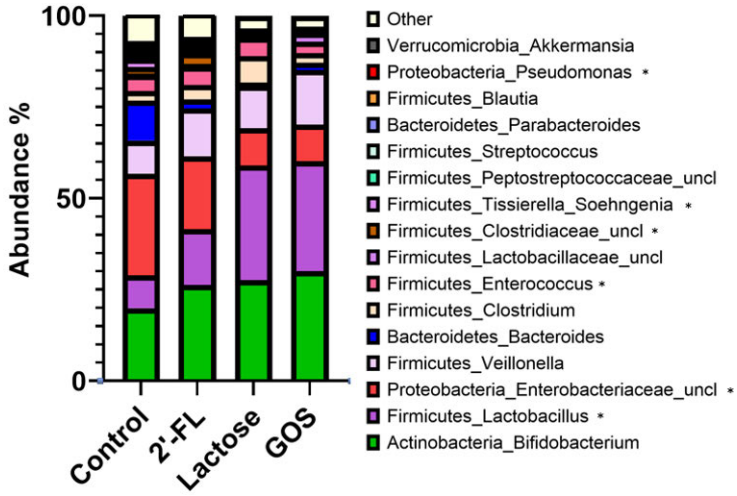

Figure 14. The relative abundances of microbiota, at the genus level, for 2´-fucosyllactose (2´-FL), lactose, and galacto-oligosaccharides (GOS), compared with the control simulation. Bacteria with relative abundances below 0.9% were combined into the Other category. All vessels were combined within each treatment group. * indicates significance $p < 0.05$ [Kruskal-Wallis tests, with Benjamini-Hochberg false-discovery rate (FDR) adjustments].

At the genus level, common changes observed for the 2´-FL, GOS and lactose treatments, compared with the control, included a significantly reduced abundance of two minor genera, *Achromobacter* and *Pseudomonas.* The primary changes that

were observed in the microbiota compositions, according to treatment, are shown in Fig. 14. Generally, 2′-FL shifted the microbiota in the same direction as GOS and lactose, but the effect was smaller for 2′-FL.

The effect of inocula donor feeding type on the microbiota composition at the genus level was also assessed by dividing the simulations into BF and FF groups. *Akkermansia*, unclassified Clostridiaceae, *Lactobacillus, Citrobacter* and unclassified Peptostretococcaceae were significantly more abundant in the FF group, whereas *Streptococcus* was more abundant in the BF group. *Prevotella* and *Peptoniphilus* were detected in BF samples but not in FF simulation samples.

The effects of microbial compositions were examined by grouping the simulations according to the rate of 2′-FL fermentation, which showed that the 2′-FL slow-fermenting group had a significantly higher abundance of unclassified Enterobacteriaceae, *Enterococcus, Achromobacter* and *Citrobacter,* whereas *Coprococcus* and *Ruminococcus* were more abundant in the 2′-FL fast-fermenting simulations. The 2′-FL fast-fermenting group also showed a trend toward the higher abundance of *Bifidobacterium* (FDR-corrected $p = 0.05$).

5.7.5 Microbial metabolites

The results regarding the production of microbial metabolites are described below.

5.7.5.1 SCFAs, lactic acid and BCFAs

The levels of SCFAs (acetic acid, propionic acid and butyric acid), lactic acid and BCFAs (isobutyric acid, 2-methylbutyric acid and isovaleric acid) in the simulation samples are shown in Fig. 15. Valeric acid was only found in 11.8% of the samples and was not examined further.

Overall, 2′-FL, lactose and GOS increased the total concentration of SCFAs and lactic acid compared with the control simulations. The differences between treatments were primarily observed in the levels of acetic and lactic acids (Fig. 15a). For 2′-FL simulations, acetic acid was the main metabolite produced, followed by lactic acid and propionic acid. In contrast, lactic acid was the main metabolite product from lactose and GOS simulations, followed by acetic acid and propionic acid. Only small amounts of BCFAs were produced. These changes were primarily due to differences in the colonic model stages in which BCFAs were detected. Control simulations showed an increase in BCFAs from V1–V4. In lactose and GOS simulations, small amounts of BCFAs were found in vessels 1 and 2, after which the levels increased in vessels 3 and 4. BCFA levels in the 2′-FL simulations were between those of the control and lactose and GOS simulations (Fig. 15b).

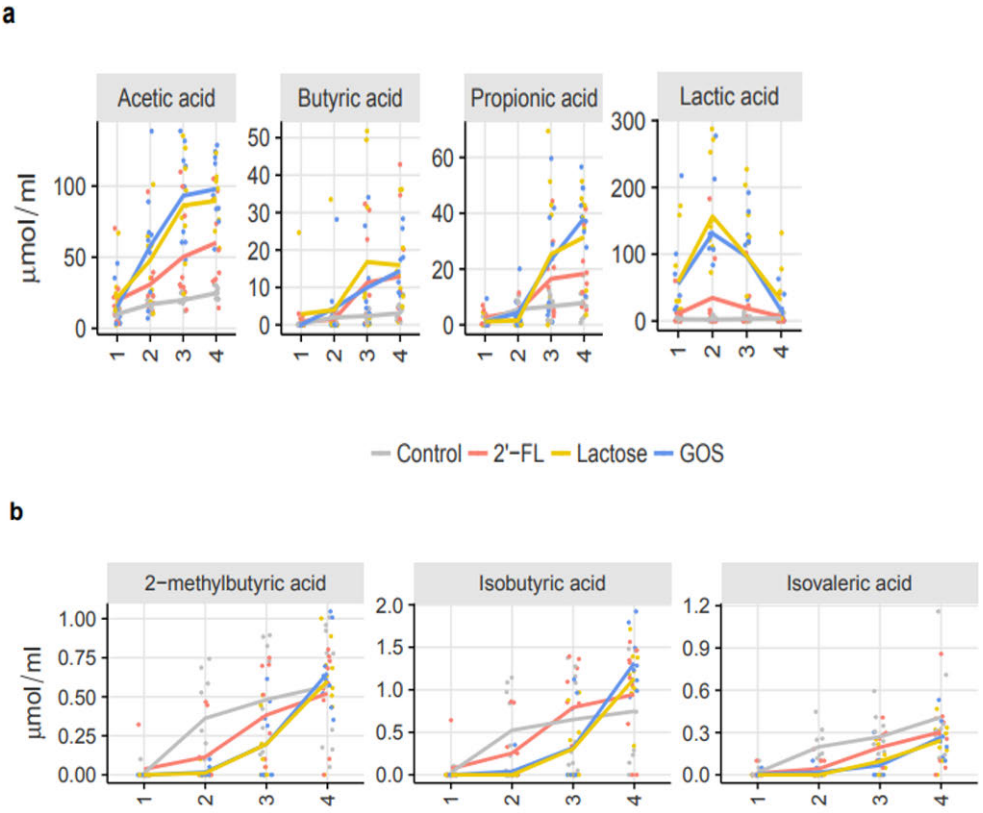

Figure 15. Smoothed averages of the treatment effects on short-chain fatty acids, lactic acid and branched-chain fatty acids, for all nine simulations. Dots represent the measurements from individual simulations. (Reproduced from the original publication IV: Salli *et al.* 2019, Sci Rep, Under Creative Commons Attribution 4.0 License, Supplementary Figure S5 a, b. Valeric acid omitted). 2´-FL = 2´-fucosyllactose, GOS = galacto-oligosaccharides.

When the simulations were divided into BF and FF groups, lactose and GOS (with higher acetic and lactic acid levels) showed increased the sum of SCFAs and lactic acid in the BF group compared with the FF group. In the FF group, 2´-FL, lactose and GOS showed significant increases in total SCFAs and lactic acid, compared with the control simulations. In both the BF and FF groups, for the lactose and GOS simulations, BCFAs were primarily detected in vessels 3 and 4.

Grouping simulations according to differences in 2´-FL utilisation, revealed that 2´-FL, lactose and GOS in the fast-fermenting group increased the total SCFA and lactic acid levels, whereas only lactose and GOS showed this effect in the slow-fermenting group. In the fast-fermenting group, 2´-FL and GOS increased acetic acid levels. BCFAs levels were lower than those in the control group for 2´-FL, lactose and GOS in the fast-fermenting group.

5.7.5.2 Biogenic amines

A total of 11 biogenic amines were measured; however, the results for β-phenyl-ethylamine, 2-methyl-butylamine and histamine were not reported because they were only detected in a few samples. Ethylamine was only detected in the inocula and simulation samples from BF donors. Fig. 16 shows 8 biogenic amines, in nine simulations, divided according to treatment. No significant changes were observed for total levels of biogenic amines between the control and treatment groups. When individual biogenic amines were examined, GOS and lactose simulations showed reduced putrescine levels compared with control simulations.

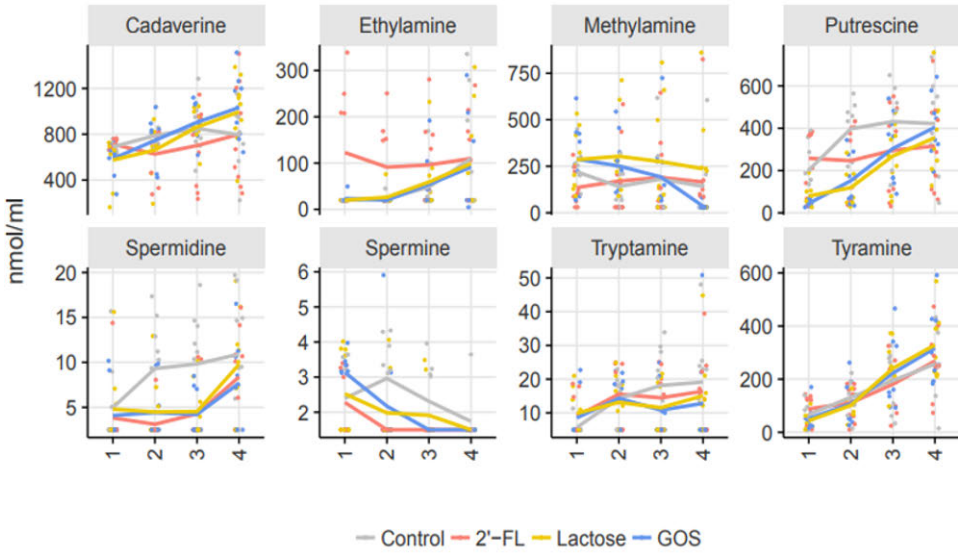

Figure 16. Smoothed averages showing the treatment effects on biogenic amines, for all nine simulations. Dots represent the measurements from individual simulations. (Reproduced from original publication IV: Salli *et al.* 2019, Sci Rep, Under Creative Commons Attribution 4.0 License, Supplementary Figure S5c). 2´-FL = 2´-fucosyllactose, GOS = galacto-oligosaccharides.

Only small differences were observed when the simulations were divided into BF and FF groups. In the BF group, compared with the BF control simulation, lactose simulations decreased the overall total level of biogenic amines, whereas 2´-FL changed the biogenic amine production profile. In contrast, for the FF group, the only change observed was for the production profile of the GOS simulation.

When the simulations were grouped according to the 2´-FL fermentation type, no changes in the biogenic amines were observed between the groups, only small changes in individual biogenic amines were observed for the GOS simulation.

6 Discussion

This thesis is composed of four *in vitro* studies. The use of *in vitro* experiments is essential to scientific research, both as a base for the design of animal and clinical experiments and to gain insights into the mechanisms of action for drugs, nutrients or other studied compounds. However, the interpretation of the results requires careful consideration because even advanced *in vitro* model system represent simplifications of *in vivo* systems. Thus, *in vitro* results should always be confirmed in preclinical or clinical settings.

6.1 *Streptococcus mutans* growth, adhesion and biofilm formation in the presence of xylitol and 2´-FL (I-III)

To our knowledge, our study was the first to report the effects of an HMO, 2´-FL, on caries-associated oral bacteria. Evaluating the effects of prebiotics and other food components is important from the perspective of oral health, especially during infancy, when the consumption of breast milk or formula occurs very frequently and the oral microbiota is still developing. Breast milk is the first nutrient received by an infant, and differences in the microbiota compositions and caries risks have been reported between BF and FF infants (Avila *et al.*, 2015; Holgerson *et al.*, 2013). Some components of breast milk (e.g. casein, lactoferrin and IgA) have already been studied with regard to the oral microbiota (Allison *et al.*, 2015; Danielsson Niemi *et al.*, 2009; Vacca-Smith *et al.*, 1994).

We evaluated the effects of the most abundant individual HMO, 2-FL, on planktonic *S. mutans* growth, using a batch model (growth for 24 h in TSB, a relatively rich media). We found that the studied *S. mutans* strains, DSM 20523, Ingbritt and CI 2366, were unable to utilise 2´-FL as a sole carbon source. In contrast, all strains grew well in the presence of GOS and lactose, whereas xylitol inhibited the growth of planktonic *S. mutans.* The inhibition of planktonic *S. mutans* growth by xylitol has been reported, using both lower (Söderling *et al.*, 2008) and higher xylitol concentrations (Misra *et al.*, 2012; Mäkinen *et al.*, 2005). In addition, the results in the present study for GOS and lactose were consistent with those reported in the existing literature (Moye *et al.*, 2014; Ooshima *et al.*, 1988; Zeng *et al.*, 2010).

2′-FL consists of fucose attached to lactose, through an α-1,2 linkage (Bode, 2012). For bacteria to utilise 2′-FL, they require an enzyme, α-1,2 fucosidase, which can cleave fucose from lactose (Sakanaka *et al.*, 2019). HMO-utilising bacteria, such as *B. longum* subsp. *infantis, B. bifidum* and some *Bacteroides* spp., have an extensive set of genes designed to hydrolyse glycosidic bonds, transport different carbohydrates across bacterial membranes, and bind specific carbohydrates (Marcobal & Sonnenburg, 2012). Geographical differences in the abundance of genes intended for the utilisation of HMOs and GOS have also been reported, in addition to differences in the abundance of typical infant bifidobacteria (Sakanaka *et al.*, 2019). In FF infants from the United States, the abundance of *Bifidobacterium* correlated with genes associated with GOS transport, whereas in BF infants from the United States, the abundance of *Bifidobacterium* correlated with genes for extracellular HMO digestion (Sakanaka *et al.*, 2019). In contrast, among BF infants from Malawi and Venezuela, the abundance of *Bifidobacterium* correlated with genes for intracellular HMO digestion (Sakanaka *et al.*, 2019). Although *S. mutans* is capable of metabolising various carbon sources (Abranches *et al.*, 2018; Moye *et al.*, 2014; Zeng *et al.*, 2010), this species appears to lack the enzymes necessary to degrade 2′-FL. The utilisation of other HMOs by oral bacteria requires further research, as the structures of HMOs vary considerably.

First the acquired enamel pellicle forms on the tooth surface (Siqueira *et al.*, 2012). The adhesion of bacteria to the tooth surface is the next requirement for biofilm formation. *S. mutans* adheres to tooth surfaces using both sucrose-independent and sucrose-dependent means (Abranches *et al.*, 2018; Banas, 2004). We wanted to evaluate whether 2′-FL, GOS, xylitol and lactose influenced the adhesion process. We studied both the adhesion to saliva-coated hydroxyapatite, reflecting the initial adhesion to salivary components within the pellicle, and polysaccharide-dependent adhesion to a glass surface, in the presence of sucrose (Haukioja *et al.*, 2008; Mattos-Graner *et al.*, 2000). During the latter adhesion model, the incubation time was 18 h, allowing bacteria to both adhere and form a biofilm. In the saliva-coated hydroxyapatite adhesion experiments, we used parotid saliva from two donors with similar adhesion-promoting properties, as parotid saliva can differ in its adhesion-promoting properties among individuals. The adherence of *S. mutans* Ingbritt to parotid saliva-coated hydroxyapatite showed huge interindividual variances, ranging from 3%–81%, among 41 participants, and the changes in adherence were not associated with differences in *S. mutans* numbers (Carlén *et al.*, 1996). HMOs have been suggested to inhibit the adherence of some pathogens to the colonic epithelium; however, these interactions and inhibitory effects are HMO-structure- and bacterium-specific (Akkerman *et al.*, 2019; Bode, 2015; Newburg, 2005). To our knowledge, the effects of HMOs on the adherence of oral bacteria have not yet been studied. We chose to examine a 1% (w/v) concentration for all

studied compounds because this is similar to the concentration of combined HMOs in breast milk, although this represents a higher concentration than can be observed for any individual HMO (Thurl *et al.*, 2017).

In our study, two *S. mutans* strains, DSM 20523 and Ingbritt, showed no significant differences in their adhesion abilities to parotid saliva-coated hydroxyapatite in the presence of any of the studied compounds, whereas the adherence of strain CI 2366 was decreased by the presence of 2′-FL and GOS. Strain-dependent differences were previously reported for *S. mutans* and *S. sanguinis* for adherence to saliva-coated hydroxyapatite and the adhesion of other oral streptococci to hard surfaces (Dorkhan *et al.*, 2012; Rosan *et al.*, 1982). When we evaluated sucrose-dependent adhesion, strain-dependent differences were observed. Here, the adhesion of the type strain DSM 20523 decreased in the presence of 2′-FL, GOS and lactose, whereas the strain CI 2366 decreased in the presence of GOS. No differences were observed for the strain Ingbritt. The decreased adhesion of *S. sobrinus* 6715 and *S. mutans* MT8148R in the presence of GOS was studied previously, using a similar experiment (Ooshima *et al.*, 1988). The effect of 2′-FL on *S. mutans* polysaccharide-mediated adhesion has not been previously reported.

We found no effects for 1% xylitol in the saliva-coated hydroxyapatite adhesion tests, a result that has not been previously reported. Xylitol binds to water molecules and forms complexes with polyvalent cations, such as Ca^{2+}, but is inert in other aspects (Mäkinen, 2010). Thus, the chemical properties of xylitol could, at least in theory, suggest that it might interfere with the saliva-coated hydroxyapatite adhesion test. Earlier studies reported decreased adhesion to a glass surface for 4% xylitol when using the same *S. mutans* strains we used, for 6% xylitol using the strain DSM 20523 and for 10% xylitol using the strain ATCC 31989 (Park *et al.*, 2014; Söderling *et al.*, 1987; Söderling & Hietala-Lenkkeri, 2010). The concentration we used was smaller than those used in previous studies, suggesting that a 1% xylitol concentration may be too low to inhibit polysaccharide-mediated adhesion in the experimental conditions used. This concentration was chosen as a comparable concentration as the concentrations of 2′-FL, GOS and lactose used in other experiments.

We have presented a continuous-culture, biofilm model for studying the early steps of dental biofilm formation. The effects of increasing xylitol concentrations, both with and without sucrose, on *S. mutans* biofilm formation were studied. A concentration as low as 2% xylitol in AS was able to reduce both biofilm and planktonic *S. mutans* numbers, which agrees with the results reported by similar studies examining planktonic *S. mutans* (Söderling *et al.*, 2008; Söderling & Hietala-Lenkkeri, 2010) and biofilm *S. mutans* (Marttinen *et al.*, 2012). The addition of 1% sucrose, together with xylitol, at xylitol concentrations between 2% to 5%, was also evaluated. The concentrations of xylitol in the saliva after the consumption of xylitol

products peaks as high as 9% and remains above 1% for 3 to 15 min after the use of the product (Lif Holgerson *et al.*, 2006; Tapiainen *et al.*, 2002). The presence of carbohydrates in combination with xylitol might represent a more realistic condition, increasing the exopolysaccharide production by *S. mutans* (Bowen & Koo, 2011; Koo *et al.*, 2010). A decrease in *S. mutans* numbers with increasing xylitol concentrations was observed also with 1% sucrose, for biofilm bacteria.

We further examined the differences between *S. mutans* strains and *S. sobrinus*. The type strain of *S. mutans*, two clinical isolates and one *S. sobrinus* strain were evaluated. The common features for all four strains in the experimental set up included an increase in biofilm bacterial numbers in the presence of 1% sucrose and a decrease in the presence of 2% xylitol, except for strain CI 117. In another model, the continuous real-time monitoring of biofilm formation showed that the addition of 0.1% to 1% sucrose increased the biofilm formation of *S. mutans*, with the strain CI 2366 being more affected by increasing sucrose concentrations than the strain DSM 20523 (Gutiérrez *et al.*, 2016). Here, differences between the studied strains were primarily identified combining 2% xylitol with 1% sucrose. The bacterial numbers of the strain CI 2366, which is known to adhere and form biofilms on glass surfaces (Söderling & Hietala-Lenkkeri, 2010), were significantly reduced by the combined use of 2% xylitol and 1% sucrose, for both biofilms and in planktonic AS, which suggested that xylitol impairs the properties associated with *S. mutans* colonisation on hydroxyapatite. Sucrose increased the biofilm formation for all four MS strains, and the effects of sucrose and xylitol on *S. sobrinus* DSM 20381 were similar to those observed for the *S. mutans* type strain.

The same model system was used to separately evaluate the cariogenicity of xylitol and sucrose mint pastilles and their main ingredients, using *S. mutans* DSM 20523. In addition to the sweetener, either xylitol or sucrose, the mint pastilles also contained PO. The effects of xylitol and sucrose were similar to those reported in original publication I; sucrose and sucrose pastilles promoted, whereas xylitol and xylitol pastilles impaired, *S. mutans* growth in planktonic AS and the numbers of bacteria in biofilms. In our experimental model, we did not observe any effects for PO alone on *S. mutans* growth or biofilm formation compared with controls. We evaluated three commercial mint oils and pooled the results because no differences were found among the studied POs. The available results regarding the antimicrobial effects of PO against *S. mutans* at the time of original publication II were controversial (Chaudhari *et al.*, 2012; Da Silva *et al.*, 2012; Rasooli *et al.*, 2008). Recent literature has indicated anti-*S. mutans* effects for PO; however, the effects are often observed when using longer adherence and growth (both planktonic and biofilm) times than those used in our study (Raghavan *et al.*, 2018; Shafiei *et al.*, 2016; Shafiei *et al.*, 2020). Likewise, the concentration of PO is important, and our goal was to use similar levels as were used in the mint pastilles. Comparisons against

other studies are also complicated because POs are often extracted from plant leaves using variable methods, which may affect their exact compositions and, therefore, affect study outcomes.

6.2 2′-FL, GOS and lactose in infant colon simulator (IV)

The development of the microbiota during infancy is affected by various factors (Zhuang *et al.*, 2019). To better understand some of these factors, especially the effects of dietary carbohydrates, the existing EnteroMix colon model was used to study changes in the infant colonic microbiota composition and the metabolites produced. We performed nine simulations using infant faecal samples as the inocula (donors were aged between 0.5 and 8 months). We found considerable variations, especially for the microbiota compositions among the simulations, due to the infant donors. Two currently available studies that focus on HMOs in infant colon models have also faced this challenge, even though the age range of their donors was smaller than ours and they used fewer donors, two infants aged six months (Wiese *et al.*, 2018) and three infants aged six months (Van den Abbeele *et al.*, 2019). Phylogenetic microarray analysis of faecal samples from three-month old Finnish infants showed relatively high interindividual variations for microbial compositions (Korpela *et al.*, 2017). The diets of the donor infants shape their microbiota compositions. Our study included both BF and FF infants and some infants who had already started eating solid foods. In contrast, other study populations were formed of exclusively BF (Wiese *et al.*, 2018) or exclusively FF infants, who had started solid foods two months prior to faecal sample donation (Van den Abbeele *et al.*, 2019).

Firmicutes was the main bacterial phylum identified in both our study and the only other *in vitro* colon study examining 2′-FL (Van den Abbeele *et al.*, 2019); however, the Actinobacteria abundance was lower in the baby-SHIME model (below 10%) than in our model. Despite the challenges associated with interindividual variability, common features were detected among the nine simulations. 2′-FL, GOS and lactose treatments decreased the abundance of the potentially harmful minor genera *Achromobacter* and *Pseudomonas* (De Weerth *et al.*, 2013; Jiang *et al.*, 2019). GOS and lactose increased *Lactobacillus* and decreased unclassified Enterobacteriaceae. When the simulations were grouped into BF and FF according to the faecal sample donors, only minor changes were observed in microbiota compositions, which is likely due to the relatively small number of simulations. The main differences observed were the increased abundance of *Lactobacillus,* unclassified Clostridiaceae and *Akkermansia* in FF samples. Thus, we also grouped the simulations according to the 2′-FL contents remaining in the vessels after the

simulations. Three simulations had no or very little 2′-FL remaining in V2, V3 or V4, indicating the presence of specific bacteria that are capable of metabolising 2′-FL. This group, which was classified as the 2′-FL fast-fermenting group, had significantly increased levels of Actinobacteria and a trend toward increased *Bifidobacterium* compared with the other simulations. In addition, the 2′-FL fast-fermenting group showed the increased abundance of *Lactobacillus*, *Coprococcus* and *Ruminococcus* and the decreased abundance of unclassified Enterobacteriaceae, unclassified Clostridiaceae and *Enterococcus*. Explaining all of these changes better would require a more detailed method for determining the compositions of microbiota, preferably to the sub-species level. Using our current 16S rRNA sequencing pipeline, we were unable to obtain this depth. In addition, the qPCR assay used in the present study was *Bifidobacterium*-specific, not species or sub-species specific. The current results highlighted the selectivity of 2′-FL fermentation by distinct bacteria compared with lactose and GOS, which can support the growth of a wider range of microbes.

Overall, the production of SCFAs and lactic acid was found to be the highest with lactose and GOS. 2′-FL was intermediate between these carbon sources and the control. These differences were primarily due to the higher production of acetic, propionic and lactic acids in the presence of lactose and GOS. Similarly, an earlier batch fermentation experiment using infant faecal material found more acetate and lactic acids produced in the presence of GOS compared with 2′-FL (Vester Boler *et al.*, 2013). Also, both GOS and 2′-FL both produced more of these metabolites than the other studied substrates (lacto-N-neotetraose, 6′-sialyllactose, gum Arabic and inulin) (Vester Boler *et al.*, 2013). In accordance with an earlier study (Vester Boler *et al.*, 2013), in this study, the FF donor simulations produced more SCFAs and lactic acid than the BF donor simulations. Controversially, other studies have found more SCFAs produced by fermentations with faecal inocula from BF than those from FF infants, *in vitro* (Parrett & Edwards, 1997). Our earlier colon simulation experiment using GOS and lactose combined with an inoculum from adult donors did not find such an increase in lactic acid production (Mäkeläinen *et al.*, 2010b; Mäkivuokko *et al.*, 2006); however, the *Lactobacillus* numbers were also lower with GOS than in the control (Mäkeläinen *et al.*, 2010b). The reasons for increased lactic acid levels in infants may be associated either with increased lactic acid production, by lactate-producing bacteria, the decreased utilisation of lactic acid, by lactate-utilising bacteria or differences in the intestinal absorption of lactic acid (which cannot occur in the model system) (Pham *et al.*, 2016). Thus, the differences in lactic acid levels observed in the current study and adult colon simulations might indicate differences in the cross-feeding of lactate between infant and adult microbiota. While less well-studied than adult lactate-utilising bacteria, *Veillonella* has been suggested to act as the main lactate-utilising bacteria in infants (Pham *et al.*, 2016).

The overall production of BCFAs (2-methylbutyric acid, isobutyric acid and isovaleric acid) was small in the simulations. Treatment with GOS and lactose significantly decreased total BCFA levels, whereas 2′-FL decreased the levels but did not reach significance, along the whole length of the colon model. The levels of total BCFAs were similar to those reported for the baby-SHIME (Van den Abbeele *et al.*, 2019). Similarly, the sum of biogenic amines was not affected by treatments, although putrescine production was decreased with GOS and lactose. When comparing biogenic amines between BF and FF samples, ethylamine was only found in the inocula or simulation samples of infants who were BF. A recent study indicated that breast milk has a higher amine content than formula, and breast milk amines increased during the first month of life; however, 2-week FF infants had higher amine levels in their stool samples than BF infants (Suárez *et al.*, 2019). Knowledge regarding BCFAs and biogenic amines, especially their effects on infant physiology and the development of microbiota, remains scarce; thus, no definite conclusions can be made.

6.3 The strengths and limitations of the dental and colon simulators

To mimic the conditions found in the oral cavity, our model used AS (Björklund *et al.*, 2011) as a growth medium. Often, BHI is used as a culturing medium for *in vitro* experiments that study MS, including biofilm formation (Park *et al.*, 2019; Söderling & Hietala-Lenkkeri, 2010). However, BHI is a rich medium; thus, the growth observed in BHI is not comparable to the growth that occurs in human saliva (Park *et al.*, 2019; Wong & Sissons, 2001). The composition of human saliva varies, and many models, including the one used here, require large volumes of saliva, making AS a more feasible choice. Our AS was mucin-based, and *S. mutans* has high proteolytic activity (Kindblom *et al.*, 2012) enabling the utilisation of nutrients from AS. Human whole saliva was used to form the pellicle and to provide natural adhesion sites for bacteria before the simulation began. The continuous flow of AS and mixing contributed to fluid shear forces, and constantly diluting the bacteria and studied compounds. In contrast to our model, many other oral biofilm model systems, which have been described previously, are batch systems (Badet *et al.*, 2008; Decker *et al.*, 2014; Giertsen *et al.*, 2011; Marttinen *et al.*, 2012). The biofilm formation times used in experiments is often much longer than in our experiment, ranging from 24 h to 5 days (Badet *et al.*, 2008; Decker *et al.*, 2014; Giertsen *et al.*, 2011), with 8 h being the closest to our model (Marttinen *et al.*, 2012). We chose a relatively short biofilm formation time to reflect the normal oral hygiene procedures, which feature frequent biofilm removal. It may also be easier to alter the properties of a young, developing biofilm. Because we focused on young biofilm formation,

our model system functions in aerobic conditions. The primary limitation of our current model was the use of a single bacterial species, preventing interactions between different bacteria. Other models that utilise 3-6 species more accurately reflect this aspect (Badet *et al.*, 2008; Giertsen *et al.*, 2011; Marttinen *et al.*, 2012). In the current study, the aerobic conditions were relevant to study the formation of a young biofilm. However, longer biofilm incubation periods would likely require the transformation of the environment to anaerobic conditions. *S. mutans* is highly competitive in a biofilm and can form biofilms as a single species, growing well under both aerobic and anaerobic conditions.

Sampling from the colon is invasive, and the use of *in vitro* models provides an ethical method to study colonic contents. However, these models have inherent limitations, such as the absence of host cells and the use of end-point faecal material as a starting point for fermentation (Pham & Mohajeri, 2018). Yet, these models also enable samples to be collected from various sections of the simulated colon and metabolites can be measured that would normally be absorbed by the human host, *in vivo.* The EnteroMix colon model includes four compartments, modelling fermentation from the proximal to the distal colon (Mäkivuokko *et al*., 2005, 2006). The main benefits of EnteroMix model include small working volumes, (it requires less test compounds than some other models), short running time and a possibility to run up to seven test compounds and a control, using the inocula obtained from a single donor. However, the acquisition of sufficient starting material from infants is considerably more challenging than collecting material from adults; therefore, in this study, we evaluated 2´-FL, GOS and lactose, in addition to the control (only artificial ileal fluid, without added carbon sources), in parallel. To accomplish this study, we used frozen faecal materials, to allow parents the time to collect sufficient faecal material for four units. Some other model systems have combined faecal materials from multiple donors (Minekus *et al.*, 1999) which has been suggested to affect cross-feeding (Van de Wiele *et al.*, 2015). Other studies, similar to ours, used only one donor at a time. In previous studies using adult faecal inocula, the variations due to donors have been relatively small (Mäkeläinen *et al.*, 2007, 2010a, 2010b; Mäkivuokko *et al.*, 2005, 2006, 2007).

6.4 General discussion

Both in the oral cavity and in the gastrointestinal tract, host-bacteria interactions are affected by the quality and quantity of dietary components. Humans are exposed to various dietary components, throughout their lives. Sweeteners, such as sucrose and other mono-, di- and oligosaccharides, are commonly used in many food products, and their increased and frequent consumption increases the risk for dental caries.

The goal for infant formula development is to achieve a composition and functionality that approaches breast milk as closely as possible. GOS and an HMO, 2′-FL have recently been added to infant formulas, to mimic the benefits of HMOs. Our understanding of the effects of individual HMOs and various combinations of HMOs on the gut microbiota composition continues to grow. However, the effects that HMOs may have on oral bacteria have been unexplored.

This study was the first to report that 2′-FL does not support the growth of *S. mutans*, whereas both GOS and lactose do. The studied compounds, 2′-FL, GOS, lactose and xylitol, did not have consistent effects on the sucrose-independent adhesion of *S. mutans* to saliva-coated hydroxyapatite or the sucrose-dependent adhesion of *S. mutans* to a glass surface. These results highlighted the importance of evaluating several aspects of food ingredients, including the effects on oral health. As a limitation, we only evaluated the effect of one HMO, 2′-FL, on the planktonic growth and adhesion of one oral bacterium *S. mutans.* Further studies remain necessary to evaluate the effects of other HMOs and HMO combinations on oral bacteria (including also bacteria other than *S. mutans*), to better understand the interactions between HMOs and oral health. In addition, it could be useful to evaluate bacterial growth in a biofilm and possibly conduct animal or clinical studies to verify *in vitro* experiments. In the future, it would likewise be of interest to evaluate the effect of other HMOs, prebiotics and human milk components and their combinations on commensal oral microbes or even oral probiotics such as *S. dentisani.* This could broaden the understanding on infant oral microbiota development.

The use of *in vitro* methods provides ethical and repeatable means for determining the effects of food components on various caries risk factors. In this thesis, a dental simulator model, using a mucin-based AS as the growth medium, was first validated by evaluating the effects of xylitol and sucrose on *S. mutans* growth and biofilm formation, which have been well established. The results were found to be in accordance with the existing literature. The system was then applied to the evaluation of the effects of actual products, in the form of mint pastilles and their components. A further advance of the current dental simulator would include the development of a system that would enable the evaluation of multiple bacterial species, which was not yet possible during this thesis.

Our understanding of microbiota development during infancy and the factors that affect this development continues to increase. Although clinical studies examining the safety and tolerance of individual HMOs among infants have been performed [reviewed in (Vandenplas *et al.*, 2018)], the available data regarding their effects on microbiota have been from adult population (Elison *et al.*, 2016). Very recently, the first report regarding the effects of 2′-FL and lacto-N-neotetraose on infant microbiota compositions was published (Berger *et al.*, 2020). Supplementation with

2′-FL and lacto-N-neotetraose was found to shift microbial diversity and composition towards that of the BF control group, at three months of age (Berger *et al.*, 2020). The EnteroMix colon model was used to study the effects of 2′-FL on the infant microbiota composition and the metabolites produced, compared with GOS and lactose. It was relevant to include another prebiotic and lactose as comparators. One limitation of this study was the level of classification of the simulated infant microbiota composition. We could only analyse bacteria to the genus level. It would be informative to identify bacteria in more detail, preferably to species, subspecies or even strain level. Then, we could better understand the changes in the microbiota composition due to 2′-FL, GOS and lactose.

Only one other study has evaluated other HMOs, including 3-fucosyllactose, 3′-sialyllactose, and 6′-sialyllactose, using the CoMiniGut model system, which was published the year before our study (Wiese *et al.*, 2018), and another study, comparing 2′-FL to a control group using the baby-SHIME model, was published in the same month as our study (Van den Abbeele *et al.*, 2019). Differences exist between the models, the parameters measured and the presentation of results, making direct comparisons difficult. Overall, a growing interest in the development of colonic model systems, especially for examining infant microbiota development, has emerged. In the future, using more advanced detection methods, we hopefully will be able to more precisely analyse the microbiota composition and increase more thorough understanding of the infant microbiota. In addition, knowledge of metabolite composition and the effects of infant diet is now well known. Advanced *in vitro* methods enable sampling for different parts of simulated colon and are valuable to understand better the effects on metabolite production.

7 Conclusions

This thesis utilised *in vitro* methods to evaluate the effects of xylitol, 2′-FL and GOS on oral MS and gut microbial compositions, compared against appropriate reference components, allowing the following conclusions to be drawn.

- Increasing the xylitol concentration decreased the numbers of MS in biofilm, both with and without sucrose. Early *S. mutans* biofilm formation was decreased by xylitol and xylitol mints. Xylitol inhibited the growth but not the adhesion of *S. mutans.* Thus, xylitol can be considered to promote beneficial changes in caries microbiota, based on the inhibition of *S. mutans* growth and early biofilm formation.
- 2′-FL did not support the growth of three strains of *S. mutans*, whereas GOS and lactose did. No consistent adhesion inhibition patterns were found. Thus, 2′-FL, a novel ingredient used in infant formulas, should not promote unfavourable changes in the caries microbiota.
- 2′-FL, similar to GOS and lactose, significantly increased Firmicutes. Actinobacteria was increased and Proteobacteria was decreased with 2′-FL, but these changes did not reach significance. The production of SCFAs and lactic acid by 2′-FL was intermediate between the control and both GOS and lactose. These results further suggest that infant formulas may have important effects on the formation and establishment of the infant gut microbiota.
- Infant colon and dental simulation models were useful methods for studying the effects of various food ingredients on bacterial growth, metabolite production and biofilm formation.

Acknowledgements

This thesis was a collaboration between Finnish Doctoral Program in Oral Science (FINDOS), University of Turku, Institute of Dentistry, and Global Health and Nutrition Science (GHNS) team in DuPont Nutrition & Biosciences. The thesis work was mostly conducted in the GHNS team research facilities in Kantvik during the years 2013-2019 and some parts of the work in the Institute of Dentistry. The Deans of the Institute of Dentistry, Juha Varrela and Pekka Vallittu, and the Professor of Periodontology, Eija Könönen, are gratefully acknowledged for providing the possibility to participate in the FINDOS postgraduate program. I warmly thank our current GHNS team leader Johanna Maukonen and her predecessors Markus Lehtinen and Sampo Lahtinen for providing facilities for the work in this PhD thesis. The financial support for the original studies from Danisco Sweeteners Oy is greatly appreciated. FINDOS Turku is thankfully acknowledged for the financial support helping to finalise the thesis writing.

I am deeply grateful to my supervisors Doc. Arthur Ouwehand, Doc. Eva Söderling and Assoc. Prof. Ulvi Gürsoy. Thank you for all your help, suggestions, knowledge sharing and all your time spent reading and commenting my articles and this thesis. You have been so supportive, encouraging, and you have always given new insights throughout the alternating circumstances during this PhD project.

I want to express my sincere gratitude the pre-examiners of this thesis, Doc. Maria Carmen Collado and Prof. Emeritus Claes-Göran Emilson. Thank you so much for taking the time to carefully review my thesis. Your through efforts and valuable insights have significantly improved the outcome. I also want to acknowledge Doc. Mervi Gürsoy and Doc. Reetta Satokari for being in my follow-up committee during the PhD project. Thank you for ideas and comments to improve and accomplish the thesis work and all your support.

Warmest thanks to all my other co-authors: Sofia Forssten, Johanna Hirvonen, Heli Anglenius, Ilmari Ahonen, Ashley Hibbert, Markku Saarinen and Kirsti Tiihonen. Science is truly team work and these articles would not have come to life without you. Blue Pencil Science is acknowledged for the copy editing of the thesis and most of the original publications. In addition, the people in the laboratories are gratefully recognised: in Kantvik Kirsi Stenström, Minna Eskola, Jaana Larsson-

Leskelä, Henri Ahokoski, Lauri Naski, and in Turku Katja Sampalahti and Oona Hällfors. You have taught me so many practical things and it has always been a joy to work with you!

Kirsti, thank you for all your support and help in this thesis project and all the other projects. Special thank you to Nick for initial revising of the language. Deep thanks to all the colleagues in GHNS team, past and present: Anna Ly., Anna La., Anneli, Auli, Arja, Alvin, Eeva-Kaarina, Elaine, Henna, Henna-Maria, Irma, Jenni L, Jenni R, Jatta, Jing, Julia, Kaisa, Katri, Kimmo, Laura H, Laura S, Liisa, Lotta, Maija, Markus, Mehreen, Mustafa, Neeta, Pia, Pilvi, Päivi, Raila, Reeta, Sanna, Sari, Satu, Sinikka, Sile, Tarja, Terhi, Tiina T and Tiina R. It has been a joy to work in a skilful, friendly, warm, and inspiring team. In addition, all other colleagues in DuPont Nutrition & Biosciences who have been part of these projects in one way or another are gratefully acknowledged.

A special thanks for Kaisa-Leena and Jussi, Laura and Markus, Annareetta and Mikko, Sanna-Mari and Janne and other friends & family for sharing your lives and bringing the balance between work & thesis work and other parts of life. Nina, thank you so much for proofreading the Finnish abstract.

Last but not least, Marjukka and Olli, the best siblings in the world, I wish to thank you and your families for all support and sharing joys and troubles of life (+our fields, forests and their maintenance). Mom, thank you for everything, all love, support and encouragement, but especially now all your practical help during the thesis writing. Thank you Karla for keeping me physically able to write, especially during COVID-19 period, Eerika for the musical performances to come and Samuel for all your help with the illustrations in the thesis. You are so special and important to me and I am so proud of you. Perttu, words cannot say enough for all your patience, support, help and love, during the thesis project and all the years we have known each other. I love you all and you are most important in my life.

Espoo, October 4th, 2020
Krista Salli

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UNIVERSITY OF TURKU

Painosalama Oy, Turku, Finland 2020

ISBN 978-951-29-8204-2 (PRINT)
ISBN 978-951-29-8205-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)