Improving broiler gut health by prebiotic stimulation of butyrate-producing bacteria

Celine De Maesschalck

This research was funded by 'Productschap Diervoeding'.

Printed by: University Press

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FACULTY OF VETERINARY MEDICINE
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Improving broiler gut health by prebiotic stimulation of butyrate-producing bacteria

Celine De Maesschalck

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Veterinary Science (PhD), Faculty of Veterinary Medicine, Ghent University, 2015

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TABLE OF CONTENT

LIST OF ABBREVIATIONS **7**

GENERAL INTRODUCTION 11

1. THE INTESTINAL MICROBIOTA: A COMPLEX ECOSYSTEM **13**
- 1.1 THE HUMAN INTESTINAL MICROBIOTA 13
- 1.2 THE CHICKEN INTESTINAL MICROBIOTA 21

2. BACTERIAL METABOLITES IN THE INTESTINE 26
- 2.1 PROTEIN FERMENTATION 26
- 2.2 CARBOHYDRATE FERMENTATION 32
- 2.3 LIPID METABOLISM 38

3. THE ROLE OF BUTYRATE IN INTESTINAL HEALTH **39**
- 3.1 ANTI-INFLAMMATORY FUNCTION 39
- 3.2 STABILIZATION OF MUCOSAL BARRIER INTEGRITY 41
- 3.3 PATHOGEN CONTROL 42
- 3.4 GLUCAGON-LIKE PEPTIDE-2 UPREGULATION 43
- 3.5 INFLUENCING PERFORMANCE 45

4. METHODS TO ENHANCE THE ENDOGENOUS PRODUCTION OF BUTYRATE **46**
- 4.1 PROBIOTICS 46
- 4.2 PREBIOTICS AND/OR DIETARY FIBRES 54

REFERENCES **67**

SCIENTIFIC AIMS 89

CHAPTER 1: *Faecalicoccus acidiformans* gen. nov., sp. nov. isolated from the chicken caecum and reclassification of *Streptococcus pleomorphus*, *Eubacterium biforme* and *Eubacterium cylindroides* as *Faecalicoccus pleomorphus* comb. nov., *Holdemanella biformis* gen. nov., comb. nov. and *Faecalitalea cylindroides* gen. nov., comb. nov., respectively, within the family *Erysipelotrichaceae* 95

CHAPTER 2: The effects of xylo-oligosaccharides on performance and microbiota in broiler chickens 111

CHAPTER 3: Cellulose is not an inert feed supplement for broilers and improves performance during the starter period 139

GENERAL DISCUSSION 159

APPENDIX 181
- **SUMMARY** **183**
- **SAMENVATTING** **186**
- **ABOUT THE AUTHOR** **189**
- **BIBLIOGRAPHY** **190**
- **DANKWOORD** **192**

LIST OF ABBREVIATIONS

16S rRNA	16 small subunit ribosomal RNA
AACC	American Association of Cereal Chemists
ADF	acid detergent fibre
AMP	antimicrobial peptides
AMPK	AMP-activated protein kinase
avDP	average degree of polymerization
avDS	average degree of substitution
AX	arabinoxylan
AXOS	arabinoxylan-oligosaccharides
BCFA	branched-chain fatty acid
BMI	body mass index
BSH	bile salt hydrolase
BW	body weight
BWG	body weight gain
CAGs	co-abundance groups
CAZymes	carbohydrate-active enzymes
CD	Crohn's disease
CH_4	methane
CO_2	carbon dioxide
CRC	colorectal cancer
CTAB	hexadecyltrimethyl ammonium bromide
DNA	deoxyribonucleic acid
DP	degree of polymerization
FAO	Food and Agriculture Organization
FAME	fatty acid methyl ester
FCR	feed conversion ratio
FI	feed intake
FOS	fructo-oligosaccharides
GIT	gastro-intestinal tract
GLP-1/2	glucagon-like peptide 1 or 2
GPCR	G protein-coupled receptor
H_2	hydrogen
HAP	dihydroxyacetinephosphate
HDAC	histone deacetylase
H_2S	hydrogen sulphide

HSP	heat shock protein
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IFNγ	interferon-gamma
IGC	integrated gene catalogue
IκB	inhibitor-kappa B
IL	interleukin
ISAPP	International Scientific Association for Probiotics and Prebiotics
JAK1/2	janus kinase 1 or 2
MAP	mitogen-activated protein
MUC2	mucin 2
N_2	nitrogen
NE	necrotic enteritis
NF-κB	nuclear factor-kappa B
NGS	next generation sequencing
NSP	non-starch polysaccharide
O_2	oxygen
OTU	operational taxonomic unit
PEG	polyethyleenglycol
PEP	phosphoenolpyruvate
PPARγ	peroxisome proliferator-activated receptor gamma
RNA	ribonucleic acid
RS	resistant starch
SCFA	short-chain fatty acid
SO_2	sulphur dioxide
SPI1	*Salmonella* pathogenicity island 1
SRB	sulphate-reducing bacteria
STAT1	signal transducer and activator of transcription 1
Sus	starch utilization system
TCA	tricarboxylic acid
TER	transepithelial resistance
TNF-α	tumor necrosis factor alpha
T-RFLP	terminal restriction fragment length polymorphism
TSA	trichostatin A
UC	ulcerative colitis
WG	weight gain
WHO	World Health Organization

XOS	xylo-oligosaccharides

General introduction

1. THE INTESTINAL MICROBIOTA: A COMPLEX ECOSYSTEM

The primary function of the gastro-intestinal tract (GIT) is the digestion of food and absorption of nutrients. In vertebrate hosts such as humans and chickens, the gut is the most densely populated and complex ecosystem, hosting bacteria, archaea, yeasts and filamentous fungi (Ley *et al.*, 2008; Lu *et al.*, 2003). Through the interkingdom cross-talk between the microbiota and the host, the intestinal microbiota plays a key role in numerous host metabolic, physiological, nutritional and immunological processes (O'Hara & Shanahan, 2006). As an example, the breakdown of complex food-derived substrates, and the metabolites produced during breakdown, is driven by the composition of the intestinal microbiota.

Most of our knowledge on gut microbiota comes from human studies, which have given us a better idea on how the microbiota of broilers could play a role in the intestinal health. Nowadays quite a lot is already known about the gut microbiota of chickens (Lei *et al.*, 2012; Lu *et al.*, 2003; Torok *et al.*, 2011; Wei *et al.*, 2013). One of the major differences between humans and chickens is the gut compartment in which bacterial fermentation takes place. In humans bacterial fermentation mainly occurs in the colon, while in chickens, fermentation takes place in the caeca (Lei *et al.*, 2012). In addition the composition of the chicken caecal microbiota has been shown to be even more complex than that of the human fecal microbiota (Eckburg *et al.*, 2005; Lei *et al.*, 2012; Wei *et al.*, 2013). Here we will first briefly introduce the intestinal microbiota of humans and then we will describe what already is known about the intestinal microbiota of chickens.

1.1 THE HUMAN INTESTINAL MICROBIOTA

1.1.1 Diversity throughout lifetime

The development of the human intestinal microbiota is a dynamic process that starts at birth and proceeds for several years through successive stages (Power *et al.*, 2014) (Figure 1A). The way of delivery already influences the initial colonization and early establishment. One-month-old children born via Caesarean section have a lower count of gut bacteria with fewer bifidobacteria compared to naturally delivered babies (Huurre *et al.*, 2008). This suggests that the environment is an important source for the initial colonization of bacteria. In naturally delivered babies the mothers' vaginal and intestinal microbiota have been shown to be a major source of intestinal microbes for the baby (Power *et al.*, 2014). During infancy (0-2 years), the gut microbiota is characterized by low-species diversity and high instability. The type of food influences the microbiota composition directly by providing the substrates for bacterial proliferation and

function (Guaraldi & Salvatori, 2012). In the gut of breast-fed infants, the dominant phylum *Actinobacteria* is represented by *Bifidobacterium* spp. (Harmsen *et al.*, 2000; Jost *et al.*, 2012) and the *Firmicutes* phylum is dominated principally by *Lactobacillus, Enterococcus* and *Clostridium* species (Turroni *et al.*, 2012; Voreades *et al.*, 2014). In contrast, the intestinal microbiota of formula-fed infants counts higher numbers of *Bacteroides* spp. as well as members of the family *Enterobacteriaceae* (Fallani *et al.*, 2010; Harmsen *et al.*, 2000). However, a study of Adlerberth and Wold showed only minor differences between the intestinal microbiota composition of breast- and formula-fed infants, suggesting that the modern formulas are mimicking more closely the composition of breast milk (Adlerberth & Wold, 2009). Supplementation of prebiotics to formula-fed infants affects the early development of the microbiota by increasing the number of bifidobacteria, as observed in breast-fed infants (Marques *et al.*, 2010; Oozeer *et al.*, 2013). The infant gut microbiota undergoes a shift with the introduction of solid food resulting in a more complex, but stable adult community (Koenig *et al.*, 2011; Yatsunenko *et al.*, 2012). During this second life stage, the intestinal microbiome comprises seven different bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia* and *Cyanobacteria* (Eckburg *et al.*, 2005). A recent study investigating the gut microbiota of 37 healthy adults showed that members of the phyla *Bacteroidetes* and *Actinobacteria* are significantly more stable components than those of the phyla *Firmicutes* and *Proteobacteria* and that 60% of the strains remained stable over the course of five years (Faith *et al.*, 2013). Environmentally introduced changes like dietary changes or antibiotic intake (Delgado *et al.*, 2006) or changes according to life events including puberty, pregnancy or menopause (Nicholson *et al.*, 2012) are primarily affecting the abundance but not the presence of specific microbial species (Rajilic-Stojanovic *et al.*, 2012). During adult life, butyrate-producing members of the phylum *Firmicutes* are important because they are responsible for the breakdown of indigestible polysaccharides and resistant starch (Bergstrom *et al.*, 2014; Koenig *et al.*, 2011). In the elderly, the relative stability and diversity of the microbiota decrease again, usually proportional to the state of health (Biagi *et al.*, 2010; Claesson *et al.*, 2012).

1.1.2 Diversity and abundance along the intestinal tract

Multiple human gut microbiome studies based on the 16S ribosomal-RNA-encoding gene have reported species diversity within and between individuals (Arumugam *et al.*, 2011; Eckburg *et al.*, 2005; Hayashi *et al.*, 2002; Lay *et al.*, 2005). Within individuals the microbial composition changes along the GIT, ranging from a narrow diversity and low number of microbes in the

stomach to a wide diversity and high numbers in the large intestine (O'Hara & Shanahan, 2006; Tiihonen *et al.*, 2010) (Figure 1B). Microorganisms of the genus *Helicobacter* and *Lactobacillus* are exposed to very acidic conditions in the stomach and to secreted bile salts and pancreatic juices in the proximal part of the small intestine (duodenum and jejunum) (Van den Abbeele *et al.*, 2013). Members of the class *Bacilli* (predominantly the *Lactobacillaceae* family) are significantly more abundant in the small intestine (jejunum and ileum), while member of the phylum *Bacteroidetes* and family *Lachnospiraceae* within the phylum *Firmicutes* are more prevalent in the colon (Frank *et al.*, 2007). In addition to variation in the composition of the microbiota along the axis of the GIT, surface-adherent and luminal microbial populations also vary (Eckburg *et al.*, 2005; Van den Abbeele *et al.*, 2013) (Figure 1C). Many species present in the intestinal lumen do not access the mucus layer and epithelial crypts (Swidsinski *et al.*, 2005). *Bacteroidetes* and *Proteobacteria* species are enriched in the luminal content while *Firmicutes* rather colonize the mucin layer, with *Clostridium* cluster XIVa or *Lachnospiraceae* family accounting for almost 60% of the mucin-adhered microbiota (Van den Abbeele *et al.*, 2013).

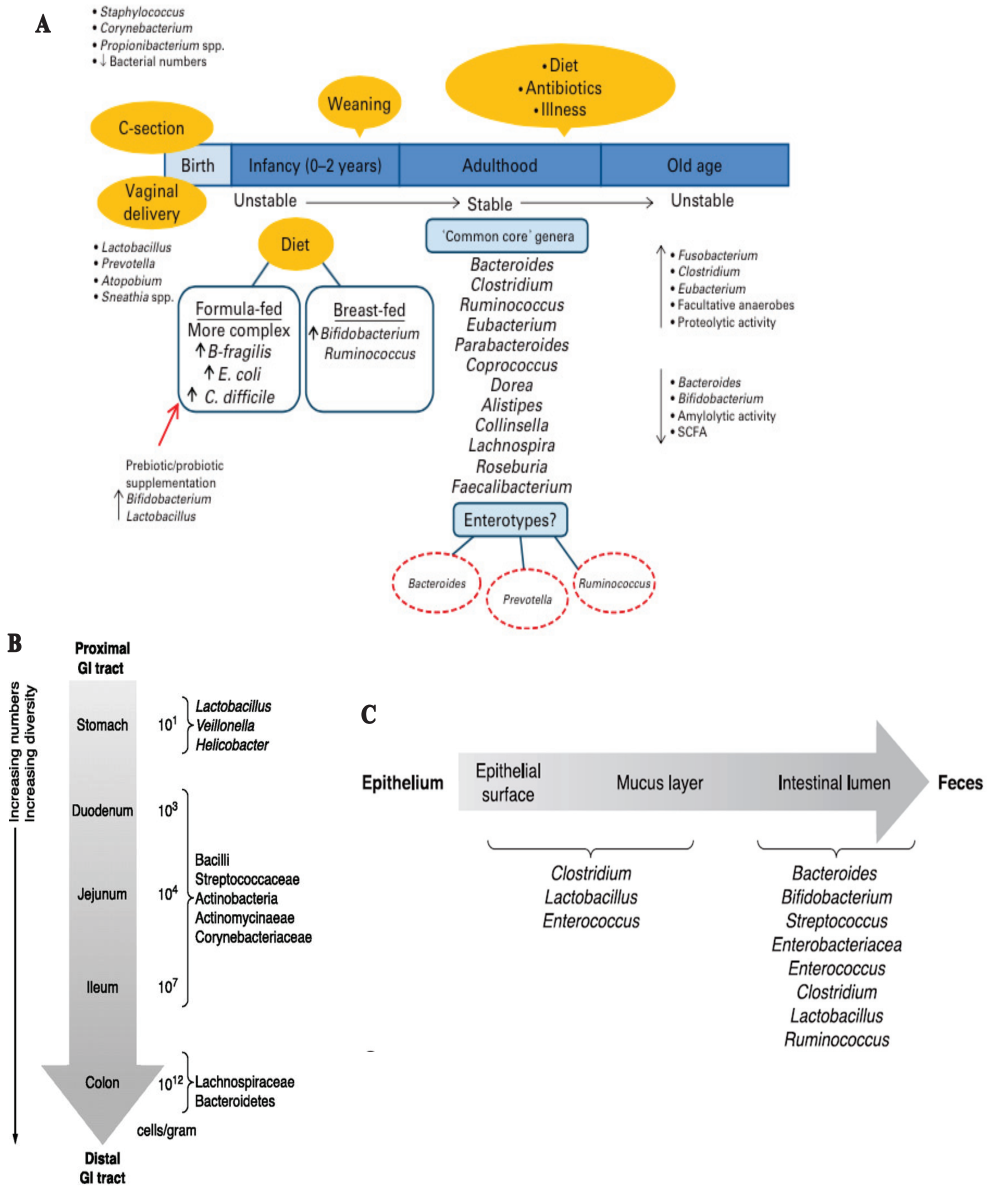

Figure 1. The human gastro-intestinal tract: A. Selected features affecting the establishment and maintenance of the microbiota and factors influencing the composition of the microbiota. C-section, Caesarean section; CFU, colony-forming units; *B. fragilis*, *Bacteroides fragilis*; *E. coli*, *Escherichia coli*; *C. difficile*, *Clostridium difficile* (Power *et al.*, 2014). **B.** Variation in microbial numbers and composition across the length of gastro-intestinal tract (Sekirov *et al.*, 2010). **C.** Longitudinal variations in microbial composition in the intestine (Sekirov *et al.*, 2010).

The species diversity between individuals was shown in a metagenomic sequencing study using 124 human fecal samples, as each individual harbored only 160 species out of 1000 prevalent bacterial species (Qin *et al.*, 2010). Almost 80% of the faecal bacteria belong either to the phylum *Firmicutes* (including *Anaerostipes*, *Ruminococcus*, *Clostridium*, *Enterococcus*, *Roseburia* and *Eubacterium* genera) or the phylum *Bacteroidetes* (including *Bacteroides*, *Prevotella* and *Alistipes* genera) (Eckburg *et al.*, 2005; Rajilic-Stojanovic *et al.*, 2007) (Figure 2A). Lower numbers of bacteria belong to other phyla like *Actinobacteria* (including the genus *Bifidobacterium*), *Proteobacteria* (including the genus *Escherichia*) and *Verrucomicrobia* (including the genus *Akkermansia*) (Arumugam *et al.*, 2011; Zoetendal *et al.*, 2008). Recently, Li *et al.* established the integrated gene catalog (IGC) of human faecal microbiome based on 1267 stool samples from individuals from three different continents. This IGC allows a rapid and multi-omic profiling of the genetic and functional repertoire of a given gut metagenome, and facilitates investigation of its geographical, genetic, temporal and physiological characteristics (Gill *et al.*, 2006; Kurokawa *et al.*, 2007; Li *et al.*, 2014; Nielsen *et al.*, 2014; Qin *et al.*, 2010). Additionally, analysis of the gut microbial communities has resulted in three predominant variants or "enterotypes", independent of host properties like nationality, sex, age or body mass index (BMI) (Arumugam *et al.*, 2011; Wu *et al.*, 2011). Enterotypes are identified by the variable level of one of three genera depending on species composition (Arumugam *et al.*, 2011) (Figure 2B-C). The first enterotype is enriched in *Bacteroides*, which mainly derives energy from carbohydrates and proteins through fermentation. The starch utilization system (Sus) in human gut *Bacteroidetes* coordinates the action of several enzymes involved in substrate binding and degradation, suggesting that closely related genera have a very broad saccharolytic potential (Martens *et al.*, 2009). Degradation of mucin glycoproteins is one of the characteristics for the second enterotype that is enriched in *Prevotella*. Bacterial enzyme activities for mucin desulphation are found in cell extracts from *Prevotella* and *Bacteroides* (Capon *et al.*, 1992; Corfield *et al.*, 1987; Rho *et al.*, 2005). Mucin-degrading *Prevotella* species co-exist with *Desulfovibrio* species and enhance the rate-limiting mucin desulphation step in mucin degradation by removing the sulphate (Wright *et al.*, 2000). *Prevotella* can also ferment more complex fibres like xylan and cellulose that are present in a diet with high concentration of carbohydrates through carbohydrate-active enzymes such as xylanases, endoglucanases and carboxymethylcellulases (De Flippo *et al.*, 2010). The third enterotype is most frequently encountered. It is enriched in *Ruminococcus* with co-occurrence of *Akkermansia*, to comprise species capable of growth on mucins as sole carbon and nitrogen source (Derrien *et al.*, 2004; Derrien *et al.*, 2010). Characteristic for species of this enterotype is the efficient binding of mucin

and its subsequent hydrolysis as well as the uptake, transport and degradation of simple sugars (Arumugam *et al.*, 2011). The phylogenetic and functional differences among enterotypes seem to reflect different combinations of microbial trophic chains (Siezen & Kleezebezem, 2011). Since methodological aspects such as clustering methodology, distance metrics, operational taxonomic units (OTUs)-picking approaches, sequencing depth, data type and 16S rRNA region all can influence the conclusions in enterotyping (Koren *et al.*, 2013), the enterotype concept needs standardized enterotyping methods to gain utility. Huse *et al.* screened 200 individuals but were unable to identify the three enterotypes. Instead they identified two 'biome types' (*Bacteroides*-*Ruminococcus* and *Prevotella*) (Huse *et al.*, 2012). A similar distribution was demonstrated in the study of Wu *et al.*, the *Bacteroides* enterotype was characterized by the additional presence of *Alistipes* and *Parabacteroides* and the *Prevotella* enterotype was characterized by the additional presence of *Paraprevotella* and *Catenibacterium* (Wu *et al.*, 2011). On the other hand the study of Claesson *et al.* introduced six co-abundance groups (CAGs) based on the dominant occurrence of members of the genus *Bacteroides*, *Prevotella*, *Ruminococcus*, *Oscillibacter*, *Alistipes* or *Odoribacter* (Claesson *et al.*, 2012). This categorization differs from the enterotypes, because here the individual microbiota is characterized by dominance of two or three CAGs rather than one of the three enterotypes. Recent analyses of human-associated bacterial diversity have categorized individuals into 'enterotypes' or clusters based on the abundance of key bacterial genera in the gut microbiota. There is a lack of consensus, however, on the analytical basis for enterotypes and on the interpretation of these results. Instead, it was recently found that rather than forming enterypes, most samples fell into gradients based on taxonomic abundance of bacteria (Koren *et al.*, 2013).

1.1.3 Diversity through diet

A number of studies suggest that enterotypes can be linked to long-term dietary patterns (Wu *et al.*, 2011). For example the *Bacteroides* enterotype has been shown to be highly associated with a diet based on animal proteins or a European diet, while the *Prevotella* enterotype has been associated with a diet with high concentrations of carbohydrates and simple sugars or an African diet (De Flippo *et al.*, 2010). Also vegetarianism alters the composition of the intestinal microbiota, resulting in a decreased number and diversity of species belonging to *Clostridium* cluster IV, XIVa and XVII (Kabeerdoss *et al.*, 2012; Liszt *et al.*, 2009). Humans in long-term residential care eating a less diverse diet have a less diverse microbiota with higher abundance of the phylum *Bacteroidetes.* In contrast, community-dwelling humans receiving a more diverse and fiber rich diet have a more diverse microbiota with higher proportion of the phylum *Firmicutes*

(Claesson *et al.*, 2012). These studies show that diet is a factor that undoubtedly influences the composition of the intestinal microbiota (Power *et al.*, 2014). These diet-associated compositional changes can lead to changes in the metabolic activity of the intestinal microbiota and the associated host response. Unfortunately, lack of detailed information and control on the diet hampers the thorough investigation of the precise effect of different dietary components on the intestinal microbiome in humans.

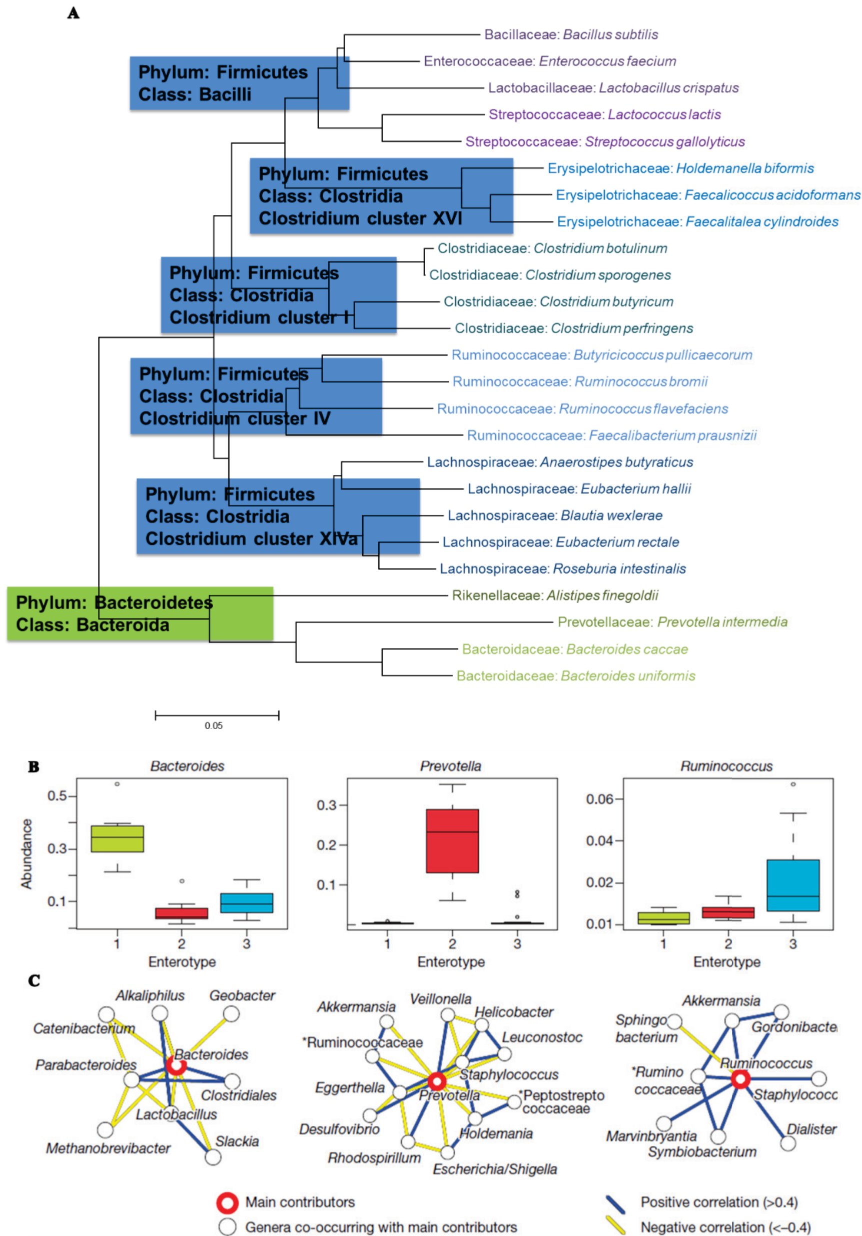

Figure 2. A. Phylogenetic tree of human gut microbiota of two most important phyla with different important classes. The phylogenetic arrangement is done on species level for different important genera of the human microbiota. **B.** Phylogenetic differences between enterotypes, abundance of the main contributors of each enterotype from the Sanger metagenomics (Arumugam *et al.*, 2011). **C.** Phylogenetic differences between enterotypes and co-occurrence networks within the three enterotypes from the Sanger metagenomes. Unclassified genera under a higher rank are marked by asterisks (Arumugam *et al.*, 2011).

1.2 THE CHICKEN INTESTINAL MICROBIOTA

1.2.1 Development and composition of the chicken microbiota

The development of the chicken gut microbiota is also a dynamic process. Although the alimentary tract of newly hatched chicks is usually sterile just like in humans, bacteria derived from the mother and the surrounding environment rapidly gain access (Amit-Romach *et al.*, 2004). Since commercial chickens are hatched in industrial hatcheries, they are exposed to a diverse range of bacteria from environmental sources such as human handlers, bedding material, feed and transport boxes, rather than from parental sources (Stanley *et al.*, 2014). Microbial colonization of the digestive tract of the chickens evolves very rapidly after hatching and achieves its functional capacity, including optimal digestion and fermentation of nutrients, during the first week (Lan *et al.*, 2005). Maximum bacterial densities are reached in the gut within the first five days post hatch and remain relatively stable, but the composition undergoes major changes (Apajalahti *et al.*, 2004). During the first four days of life the environmental conditions along the intestinal tract do not allow microbial differentiation, resulting in the dominance of facultative anaerobes like streptococci and enterobacteria in all segments of the intestine (van der Wielen *et al.*, 2002). As chickens age, changes in environmental conditions in different segments of the intestine such as pH, nutrients and oxygen tension, result in the development of a specific bacterial community for each intestinal compartment (van der Wielen *et al.*, 2002). At seven days post hatch the caecal microbiota is a subset of the ileal microbiota and is dominated by three genera (*Flavonifractor*, *Pseudoflavonifractor* and *Ruminococcus*) (Oakley *et al.*, 2014a). A typical adult gut microbiota is suggested to be established within three weeks. In the caecum the genus *Faecalibacterium* accounts for 23-55% of the identified 16S rRNA sequences according to Oakley *et al.*, while Stanley *et al.* found an abundance of *Clostridium*, *Ruminococcus*, *Eubacterium*, *Faecalibacterium* and *Lactobacillus* species in the caecum (Oakley *et al.*, 2014a; Stanley *et al.*, 2014). The establishment of the small intestinal microbiota is faster than the caecal microbiota, but the microbial community in the caeca has greater richness and diversity in comparison to the ileal community (Lan *et al.*, 2005; Shaufi *et al.*, 2015).

Chicken gut 16S rRNA gene sequences have been deposited in three public databases (Genbank, Silva and Ribosomal Database Project) (Wei *et al.*, 2013). A total of 915 species-equivalent OTUs are divided over 12 phyla and 117 established bacterial genera (Wei *et al.*, 2013). *Firmicutes* constitute the most predominant phylum accounting for almost 70% of all bacterial sequences (Wei *et al.*, 2013). *Bacteroidetes* (12.3%) and *Proteobacteria* (9.3%) are the second and third predominant phylum respectively (Wei *et al.*, 2013). Arumugam *et al.* suggested that

enterotypes also exist in animals (Arumugam *et al.*, 2011). This is confirmed by Kaakoesh *et al.*, who compared the faecal microbiota of thirty-one 56-day old chickens from two Australian farms and showed that the gastro-intestinal microbiota of the chickens can be classified into four enterotypes (Figure 3). Enterotype 1 is dominated by *Firmicutes*, enterotype 2 by *Firmicutes* and *Proteobacteria*, enterotype 3 by *Firmicutes* and *Actinobacteria* and enterotype 4 by *Firmicutes* and *Bacteroidetes* (Kaakoesh *et al.*, 2014). The enterotypes of chickens are based on phylum level while those of humans are based on genus level. When looking at lower phylogenetic level, it can be stated that enterotypes 2 and 3 are derived from enterotype 1 that is dominated by lactobacilli at genus level and *Peptostreptococcaceae* at family level. Chickens with enterotypes 2 and 3 are suggested to be later on efficiently colonized by *Escherichia*, *Shigella* and *Enterobacter* species belonging to the phylum *Proteobacteria* or *Corynebacterium* and *Brevibacterium* species belonging to the phylum *Actinobacteria*. In contrast, the *Ruminococcaceae* family dominates the *Firmicutes* from enterotype 4 and this dominance is shared with the *Bacteroidetes* genera *Alistipes* and *Bacteroides*. Recently, the existence of a *Ruminococcaceae* dominated enterotype in chickens was confirmed by Videnska *et al.* (Videnska *et al.*, 2014). No other studies have divided the microbiota of the chickens into enterotypes, which suggests that further research is necessary to confirm this classification.

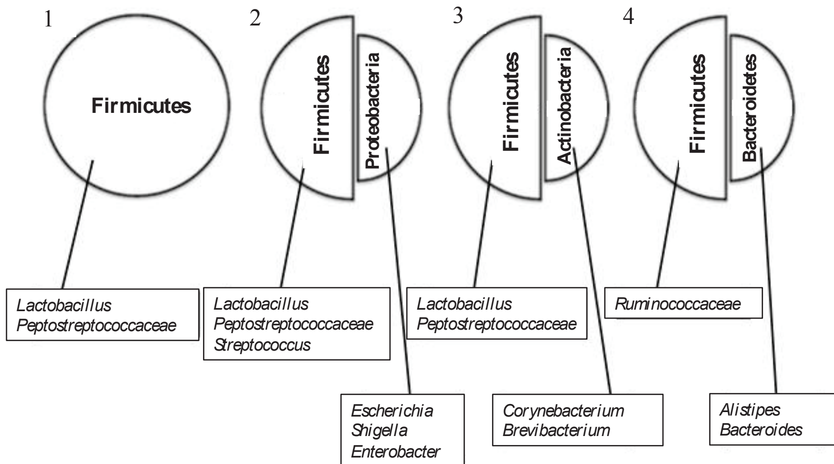

Figure 3. Taxa identified to be the highest contributors within each enterotype in broiler chickens (Kaakoesh *et al.*, 2014).

1.2.2 Impact of nutrition on the chicken intestinal microbiota

The feed composition for broilers is traditionally changed three times during their lifespan. These changes influence the microbiota composition. Broiler chickens get a starter diet from day 1 until around day 14, which determines the initial composition of the microbiota. From day 15 onwards, chickens receive a grower diet. During the last phase, which takes from day 29 until slaughter age, a finisher diet is given The feeding programs for broilers can also include 4 or 5 phases and even multiphase diets where feed is changed every seven days (Buteri *et al.*, 2009). The multiphase feeding programs are designed to meet the nutritional needs of birds more closely at specific points in their life cycle (Gutierrez *et al.*, 2008), while the traditional programs are based on average requirements and thus can provide lower or greater amounts of nutrients than those needed (Hauschild *et al.*, 2014).

It is generally accepted that the gut microbiota has an impact on avian growth and health and that the activities of the microbiota can be manipulated by altering the diet (Knarreborg *et al.*, 2002; Pan & Yu, 2014). In several studies it has even been suggested that diet might be the main contributor to differences and abundance in microbial community (Gong *et al.*, 2002a; Lu *et al.*, 2003; Zhu *et al.*, 2002). High levels of non-starch polysaccharides (NSP) as found in wheat or rye, lead to increased viscosity, decreased passage rate of the digesta, and lower nutrient digestibility, which in turn favors the growth of *Clostridium perfringens.* This bacterium is considered one of the predisposing factors of necrotic enteritis (Choct *et al.*, 1996; Timbermont *et al.*, 2011). Chickens fed a rye-based diet have increased numbers of total lactic acid bacteria in duodenum, ileum and caecum compared to chickens fed a corn-based diet (Tellez *et al.*, 2014). Torok *et al.* was the first to correlate diet-associated changes in the gut microbial community with improved performance (Torok *et al.*, 2008). This was taken a step further in another study, where three feeding trials were investigated in order to identify gut bacteria consistently linked with better broiler performance (Torok *et al.*, 2011). This study was able to identify eight OTUs from ileum or caecum linked to broiler performance using terminal restriction fragment length polymorphism (T-RFLP). Sequencing of these OTUs revealed that they might represent 26 different bacterial species or phylotypes. Some of them showed high sequence homology with *Lactobacillus salivarius*, *L. aviaries*, *L. crispatus*, *Faecalibacterium prausnitzii*, *Escherichia coli*, *Gallibacterium anatis*, *Clostridium lactatifermentans*, *Ruminococcus torques*, *Bacteroides vulgatus* and *Alistipes finegoldii* (Torok *et al.*, 2011). All these studies underline the importance of dietary influences on the intestinal microbiota affecting broiler performance.

Every chicken has its own unique dominant intestinal bacterial community and host-specific factors are suggested to be important in the establishment of that community (van der Wielen *et al.*, 2002). As feed passes through the GIT, it encounters specialized microbial communities that perform important digestive functions (Oakley *et al.*, 2014b; Stanley *et al.*, 2014) (Figure 4). The crop is dominated by various *Lactobacillus* spp. that break down starch with the formation of lactate (Rehman *et al.*, 2007). Lactobacilli also dominate the proventriculus and the gizzard. The main difference between the crop and gizzard as detected by measuring metabolic products is the lower fermentation activity in the gizzard, due to low pH (Engberg *et al.*, 2004; Rehman *et al.*, 2007). Using classic culturing methods, *Lactobacillus* spp. have been found to be dominant in the duodenum and ileum (Stanley *et al.*, 2014), together with *Streptococcus*, *Enterobacteriaceae* and various *Clostridiaceae* (Gong *et al.*, 2002b; Kohl, 2012; Pan & Yu, 2014; Stanley *et al.*, 2014). The function of the ileum is mainly nutrient absorption, while in the caecum mainly bacterial fermentation occurs (Gong *et al.*, 2002a). Since these regions function differently and provide a different environment, it is expected that they are colonized by different types of bacteria (Gong *et al.*, 2002a). The most abundant bacteria in the chicken caeca are members of the *Clostridiaceae*, *Bacteroidaceae*, *Lactobacillaceae*, *Proteobacteria*, butyrate producing clusters of *Firmicutes*, with an abundance of the genera *Clostridium*, *Ruminococcus*, *Eubacterium* and *Faecalibacterium* among a number of unknown and uncultured phylotypes (Stanley *et al.*, 2014). Beside the composition also the function of the microbiome can be affected by various factors such as dietary ingredients, nutrient level, environment, probiotic, and antibiotic treatments (Qu *et al.*, 2008).

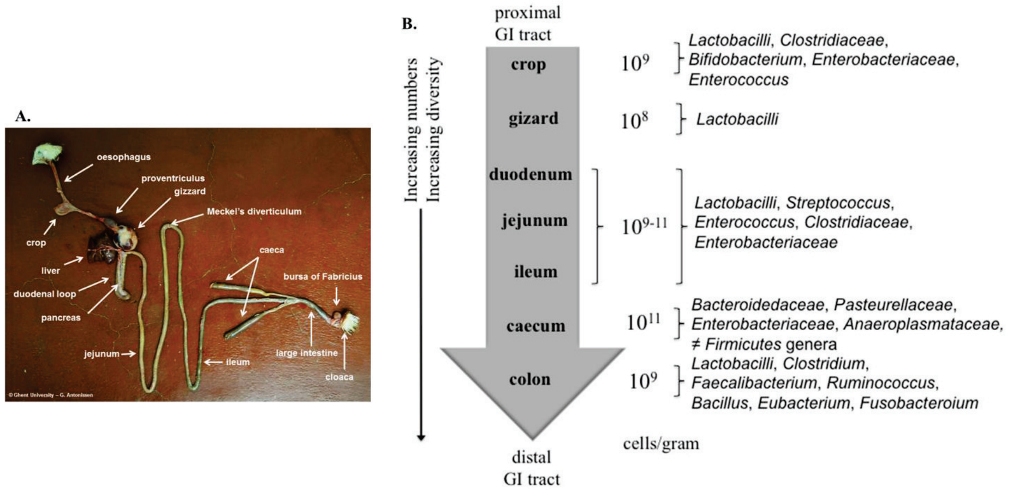

Figure 4. A. Gastro-intestinal tract of a three-week-old broiler chicken. **B.**Variation in microbial numbers and composition along the gastro-intestinal tract (GIT) of chickens (Adapted from Sekirov *et al.*, 2010).

Further insight into the gut ecosystem is needed to understand the exact role of microbiota in health and gastro-intestinal disease. Nutritional strategies to manage the composition of the intestinal microbiota, their function and thus the detrimental or beneficial outcomes, will have practical value in the future.

2. BACTERIAL METABOLITES IN THE INTESTINE

Metabolites are produced by various members of the microbial community and consumed or transformed by others. These metabolites serve different functions. They also constitute an important communication tool between the microbiota and the host immune system (Dorrestein *et al.*, 2014; Geuking *et al.*, 2013). Therefore not only the composition but also the functional capacity of the intestinal microbiota is important (Hamer *et al.*, 2012).

Studies analyzing the gut microbiome of different animal species including chicken (Qu *et al.*, 2008) have emphasized the critical role of the diet in shaping the microbial community structure. In addition, observations from reciprocal microbiota transplantation experiments are consistent with the idea that a number of selection pressures in the host act to influence the community structure during or after colonization (Gootenberg & Turnbaugh, 2011). Furthermore differences in anatomical structures between the chicken and human lower intestinal tract may affect the availability of fermentable substrates causing the specialization of the colonizing microbiota. These observations suggest that the host exerts key influences on the composition of the gut microbiome. However in both human and chicken the sequences belonging to *Lachnospiraceae* and *Ruminococcaceae* are shown to dominate (Jozefiak *et al.*, 2010; Lozupone *et al.*, 2012). Additionally Lei *et al*. revealed that human fecal microbiota share many phylogenetic groups with chicken caecal microbiota at the genus level, while at the species level significant differences may be attributed to the metabolic characteristics associated with each species (Lei *et al.*, 2012). The vast majority of papers analyzing bacterial metabolites in the intestine relate to the human microbiome and to some extent also that of lab animals, while literature specifically investigating the chicken is limited. Therefore, in this chapter the metabolic pathways reviewed are microbial group specific with information derived mostly from studying human microbiota, and results and conclusions can partly be extrapolated to the chickens.

It is generally accepted that carbohydrate fermentation results in beneficial effects for the host because of the generation of short-chain fatty acids (SCFA), whereas protein fermentation is considered detrimental for the host's health (Windey *et al.*, 2012). Particular metabolic profiles may serve as a diagnostic tool for the identification of several diseases in humans, such as ulcerative colitis (UC), with increased faecal concentration of hydrogen sulphide and Crohn's disease (CD), with a decreased concentration of SCFAs in faecal extracts (Huda-Faujan *et al.*, 2010; Ohge *et al.*, 2005; Pitcher *et al.*, 2000; Roediger *et al.*, 1997).

2.1 PROTEIN FERMENTATION

Protein fermentation results in the production of a wide range of metabolites that are in direct contact with the colonic mucosa and interact with the mucosal cells (Windey *et al.*, 2012). Degradation of proteins starts with the hydrolysis into smaller peptides and amino acids by bacterial proteases and peptidases (Windey *et al.*, 2012). Further anaerobic fermentation of aromatic amino acids such as tyrosine and tryptophan results in phenols and indoles. Oxidative or reductive deamination of the amino acids results in ammonia while decarboxylation results in different amines (Rinttila & Apajalathi, 2013) (Figure 5).

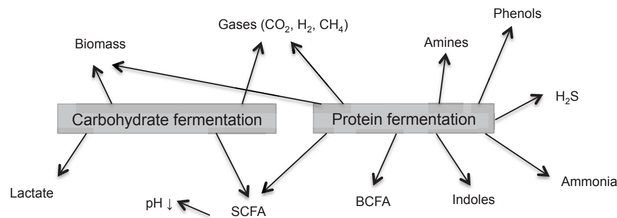

Figure 5. Different metabolites produced from colonic fermentation of carbohydrates and proteins. BCFA, branched-chain fatty acids; SCFA, short-chain fatty acids (adapted from Hamer *et al.*, 2012).

2.1.1 Phenolic and indolic compounds

Phenolic and indolic compounds are formed following bacterial degradation of aromatic amino acids such as tyrosine, tryptophan and phenylalanine (Hughes *et al.*, 2000). Different degradation products of tyrosine are phenol and *p*-cresol while tryptophan degradation generates indole and derivatives, including skatole. Bacterial metabolism of phenylalanine leads to derivatives such as phenylpyruvate and phenyllactate (Windey *et al.*, 2012). The intestinal bacteria involved in these processes include clostridia, *Bacteroides*, enterobacteria, bifidobacteria and lactobacilli (Aragozzini *et al.*, 1979; Botsford & Demoss, 1972; Chung *et al.*, 1975; Elsden *et al.*, 1976; Hughes *et al.*, 2000; Yokoyama & Carlson, 1974).

Phenol is a potential driver of gut-barrier alterations (Leclercq *et al.*, 2014). This was demonstrated in the study of McCall *et al*. who showed a dose-response effect of phenol on epithelial barrier function using transepithelial resistance (TER) and FITC-dextran permeability measurements on SK-CO15 cells, a transformed human colonic epithelial cell line (McCall *et al.*, 2009). The TER decreased with increasing concentration of phenol (1µM, 100µM, 1mM, 3.2mM, 6.4mM, 10.6mM, 15.9mM, 21.2mM) suggesting disruption of the epithelial barrier after exposure to phenol. In addition a significant increase in flux of FITC-dextran was shown,

suggesting a phenol induced increase of the increases epithelial paracellular permeability *in vitro* (McCall *et al.*, 2009). Phenol (1.25-1.5mM) has also been shown to significantly impair the viability of human colonic epithelial HT-29 cells, suggesting a direct toxic effect of phenol *in vitro* (Pedersen *et al.*, 2002).

Indole, on the other hand was shown to have a beneficial role in establishing an epithelial barrier *in vitro* and *in vivo* by inducing the expression of several genes involved in epithelial cell function (Bansal *et al.*, 2010; Shimada *et al.*, 2013). The *in vitro* study of Bansal *et al*. showed coordinated control of inflammation in the presence of 1mM indole through the repression of several inflammatory cytokines and coordinated regulation of signaling pathways (Bansal *et al.*, 2010). Germ-free mice treated with indole-containing capsules (15mg) showed a higher resistance to epithelial damage induced by dextran sodium sulphate (Shimada *et al.*, 2013). This suggests that indole promotes the stability of the intestinal epithelial barrier and has no toxic effects (Shimada *et al.*, 2013). But further hydroxylation of indole results in 3-hydroxy-indole, the majority of which is sulfonated to indoxyl sulphate, an uremic toxin (Meijers & Evenepoel, 2011; Niwa, 2010). There is a direct association between indoxyl and cardiovascular disease and overall mortality, and it is also markedly accumulated in the serum of patients with chronic kidney disease (Barreto *et al.*, 2009; Meijers & Evenepoel, 2011; Niwa, 2010).

In chickens, to our knowledge there is nothing to be found about the effects of phenol and indole. The only thing that was found were *Spirochetes* chicken isolates that were able to produce indole (McLaren *et al.*, 1997).

2.1.2 Ammonia

Ammonia is produced through oxidative or reductive deamination of the terminal amine group of the amino acids or to some extent also through urea hydrolysis catalyzed by urease activity in the distal gut (Blachier *et al.*, 2007; Hughes *et al.*, 2000; Rinttila & Apajalathi, 2013; Windey *et al.*, 2012). Large amounts of ammonia are generated by Gram-negative anaerobes, clostridia, enterobacteria, or *Bacillus* spp., while Gram-positive non-spore forming anaerobes or streptococci produce modest amounts of ammonia. Only small amounts are formed by lactobacilli or yeasts (Vince & Burridge, 1980).

The presence of ammonia (35µmol/g) in the hindgut has been shown to affect the kinetics of colonocytes including increased mucosal cell turnover, which suggests the possible involvement in tumor promotion and thus the increase in incidence of colon carcinomas (Ichikawa & Sakata,

1998; Rowland *et al.*, 1998). Another study using a rat model showed the increased production of ammonia (1-2µg) and incidence of colon cancer as a result of a high protein diet (Topping & Visek, 1976). Also ammonium acetate (24.8mg) has been shown to increase the incidence and total number of colonic carcinomas in rats (Clinton *et al.*, 1988). It has been hypothesized that this toxic effect of ammonia can be counteracted by butyrate (Bartram *et al.*, 1993; Windey *et al.*, 2012). In normal conditions ammonia is rapidly absorbed into the portal blood, converted to urea in the liver and excreted in the urine (Hamer *et al.*, 2012; Hughes *et al.*, 2000).

In the blood circulation of broiler chickens, ammonia is easily absorbed through the intestinal epithelium and has toxic effects on enterocytes (Karasawa & Nakata, 1986; Macfarlane & Macfarlane, 2012; Rinttila & Apajalathi, 2013).

2.1.3 Amines

The decarboxylation of amino acids results in the presence of different amines in the gut (Pessione *et al.*, 2005; Windey *et al.*, 2012). Amines found in the gut are produced by colonic bacteria belonging to the genera *Clostridium*, *Bifidobacterium* and *Bacteroides* and include agamatine, tyramine, pyrrolidine, histamine, piperidine, cadaverine, putrescine and 5-hydroxytryptamine (Allison & Macfarlane, 1989; Drasar & Hill, 1974; Hughes *et al.*, 2000) which are further detoxified by monoamine and diamine oxidases (Hughes *et al.*, 2000; Windey *et al.*, 2012). The most active amines are histamine and tyramine that are decarboxylation breakdown products of histidine and tyrosine, respectively (Ladero *et al.*, 2010).

Histamine binds specific receptors in the gut and results in the contraction of the intestinal smooth muscle cells and the dilatation of the surrounding blood vessels (Jarisch, 2004; Ladero *et al.*, 2010). There are no histamine effects when the plasma histamine concentration is 0-1 ng/mL (Maintz & Novak, 2007). Higher histamine concentrations (1-2ng/mL) have been associated with a number of inflammatory and neoplastic diseases such as CD, UC and colorectal neoplasms (Ladero *et al.*, 2010; Maintz & Novak, 2007). An excess of tyramine (> 6mg/kg) in the GIT can lead to its entering into the systemic circulation, through which it can reach adrenergic nerve terminals or can be β-hydroxylated to octopamine and stored in vesicles with the gradual displacement of norepinephrine, resulting in a transient increase in blood pressure (Ladero *et al.*, 2010). Indirect toxic effects have been described for putrescine and other polyamines, with a role in the regulation of cell growth, proliferation and maturation (Dufour *et al.*, 1988; Ladero *et al.*, 2010; Seiler *et al.*, 1998). Their levels (daily intake for adult vary between 350 and 550µmol) need to be tightly regulated since a disturbed equilibrium may lead to the dysregulation of certain

physiological functions such as neurotransmitter, cell growth and differentiation, gastric acid secretion, immune response, increased cardiac output, regulation of gene expression, etc. (Ladero *et al.*, 2010). This suggests that putrescine and others have a role in promoting the malignant transformation of cells (Gerner & Meyskens, 2004; Ladero *et al.*, 2010; Seiler *et al.*, 1998; Wallace & Caslake, 2001). Other amines have been linked to different diseases like migraine, hepatic coma, hypertension, heart failure, cancer and gastro-intestinal disease (Hughes *et al.*, 2000; Ladero *et al.*, 2010; Macfarlane, 1997; Murray *et al.*, 1993; Seidel *et al.*, 1984; Smith, 1981).

Amines like phenylethylamine (4.8mg/kg), putrescine (49mg/kg), cadaverine (107mg/kg), histamine (131mg/kg) or a combination of all these amines have been implicated in causing poor performance and intestinal lesions in broilers (Bermudez & Firman, 1998).

2.1.4 Sulphides

Hydrogen sulphide (H_2S) is a bacterial metabolite present in the lumen of the distal intestine, which is produced through fermentation of sulphur-containing amino acids, through the reduction of inorganic sulphate and sulphating agents such as sodium and potassium sulphite, metabisulphite, bisulphites and sulphur dioxide (SO_2) or through intestinal sulphomucin metabolism (Blachier *et al.*, 2007; Roediger *et al.*, 1997). Sulphate-reducing bacteria (SRB) are a diverse group that reduce inorganic sulphate to H_2S. Therefore, SRB are sharing the ability to use sulphate as terminal electron acceptor and H_2 as their electron donor for respiration with the concomitant production of H_2S (Carbonero *et al.*, 2012; Fite *et al.*, 2004) (Figure 6). The amounts of dietary inorganic sulphate and sulphur amino acids are critical in determining sulphate and H_2S production in the distal intestine (Hughes *et al.*, 2000).

H_2S has been shown to modulate peripheral nociceptive (pain-related) signals (Kawabata *et al.*, 2007). H_2S (1-2mmol/L) is highly toxic to colonocytes and impairs their metabolic function, especially butyrate oxidation (Roediger *et al.*, 1993a, b). The anionic sulphide concentration is elevated in the colon of patients with UC (Roediger *et al.*, 1997). Either increased sulphidogenic activity or reduced sulphite detoxification in the colonic epithelium might explain the increased H_2S concentrations (0.6mM) in UC patients and their potential inflammatory impact (Carbonero *et al.*, 2012; Ohge *et al.*, 2005; Pitcher *et al.*, 2000). H_2S can damage the intestinal epithelium leading to chronic inflammation (Babidge *et al.*, 1998; Roediger *et al.*, 1997; Roediger *et al.*, 1993a) as well as perturbation of the balance between cellular proliferation and apoptosis (Cai *et al.*, 2010; Deplancke & Gaskins, 2003; Leschelle *et al.*, 2005). The studies of Attene-Ramos *et*

al. confirmed that H_2S (> 500µM) perturbs cellular homeostasis in the colonic mucosa, affecting cell proliferation and increasing inflammation (Attene-Ramos *et al.*, 2006; Attene-Ramos *et al.*, 2010). The observed association of increased H_2S with colorectal cancer is derived from cysteine fermentation rather than sulphate respiration (Carbonero *et al.*, 2012). H_2S is toxic to microbes in general. It also helps to maintain the anaerobic status (Carbonero *et al.*, 2012).

No effect of sulphides and sulphate-reducing bacteria related to chickens are already described.

2.1.5 Branched-chain fatty acids

Branched-chain fatty acids (BCFA) are end-products of bacterial fermentation of proteins and are not produced from carbohydrate fermentation (Blachier *et al.*, 2007; Nordgaard *et al.*, 1995; Rasmussen *et al.*, 1988). Compared to previous protein fermentation products, BCFAs are not toxic to the host cell.

Little is known about the metabolism of BCFAs in colonic epithelial cells. Iso-butyrate may serve as a fuel in colonocytes when butyrate availability is defective (Jaskiewicz *et al.*, 1996). So far no measurable effect of BCFAs could be shown on proliferation, differentiation or apoptosis using *in vitro* models of colonic adenocarcinoma cells (Blachier *et al.*, 2007).

The level of iso-butyrate, valerate and iso-valerate were measured in a study that describes the effect of isomalto-oligosaccharides in broilers (Zhang *et al.*, 2003). This study showed that the levels of BCFA were decreased when the chickens were fed with isomalto-oligosaccharides. This suggested that there is indeed production of BCFA possible in chickens.

2.2 CARBOHYDRATE FERMENTATION

Under the anaerobic conditions of the distal intestine, undigested carbohydrates are fermented mainly to gases and SCFAs (Flint *et al.*, 2012a; Flint *et al.*, 2012b; Louis *et al.*, 2014; Samuel & Gordon, 2006) (Figure 5).

2.2.1 Gases

The production and metabolization of colonic gases such as hydrogen (H_2), carbon dioxide (CO_2), methane (CH_4), nitrogen (N_2) and oxygen (O_2) influences health and disease (Carbonero *et al.*, 2012). H_2, CO_2 and CH_4 are produced solely by microbes in the distal gut, which ferment dietary components that escape digestion by host enzymes and endogenous substrates derived from the distal gut mucosa (Carbonero *et al.*, 2012; Suarez *et al.*, 1997). The accumulation of gas in the colonic lumen is dependent on the interplay between various microbial metabolic pathways and host physiology. The role of gaseous by-products of microbial fermentation has been implicated in irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), obesity and colorectal cancer (CRC) (Carbonero *et al.*, 2012). The concentrations of gases were difficult to measure as they are very rapidly absorbed during passage through the intestine.

Luminal N_2 can be derived from either swallowed air or diffusion from the blood (Suarez *et al.*, 1997).

To our knowledge, there were no studies that describe directly the effect of the colonic gases in chickens. But different microbiota studies showed the presence of bacteria that are responsible for the production of gases (Lu *et al.*, 2003). For example, the study of Saengkerdsub *et al*. could successfully detect methanogenic archaea in faecal samples from 3- to 12-day-old broiler by qPCR (Saengkerdsub *et al.*, 2007).

2.2.1.1 Carbon dioxide

CO_2 is derived predominantly from bacterial fermentation (Suarez *et al.*, 1997). Most CO_2 is absorbed by the colonic mucosa and recirculates in the blood or passes as flatus (Carbonero *et al.*, 2012; Christl *et al.*, 1992).

2.2.1.2 Hydrogen

Hydrogen (H_2) can be produced by cleavage of pyruvate to formate and subsequent conversion by formate hydrogen lyase, or by generation from pyruvate through the activity of pyruvate:ferredoxin oxidoreductase and hydrogenase (Carbonero *et al.*, 2012; Macfarlane, 1997).

The production of H_2 by hydrogenogenic microbes such as *Ruminococcus* spp., *Roseburia* spp., clostridia spp. and *Bacteroides* spp., is crucial for the efficiency of fermentation and has a key role in anaerobic ecosystems (Carbonero *et al.*, 2012; Flint *et al.*, 2012a).

Bacterial H_2 production affects the metabolism of hydrogen-utilizing fermentative bacteria (Flint *et al.*, 2012a; Macfarlane & Macfarlane, 2003). Microorganisms that use H_2 (hydrogenotrophic bacteria), such as methanogenic archaea (e.g. *Methanobrevibacter smithii*), acetogenic bacteria (e.g. *Blautia hydrogenotrophica*) and sulphate-reducing bacteria (e.g. *Desulfovibrio* spp., producing H_2S), have a particularly important role in anaerobic metabolism via interspecies cross-feeding interactions (Louis *et al.*, 2014) (Figure 6). Reductive acetogens are more metabolically versatile than methanogenic archaea or sulphate-reducing bacteria (Carbonero *et al.*, 2012). The nature of the interaction of acetogens with these two groups of hydrogenotrophic microbes and the extent to which they contribute to H_2 disposal in CH_4-excretors versus non-excretors remains to be resolved (Carbonero *et al.*, 2012).

2.2.1.3 Methane

Colonic methanogenic archaea (*Methanobrevibacter smithii* and *Methanosphaera stadtmanae*) derive all (or most) of their metabolic energy from methanogenesis by reducing CO_2 or methanol to methane (CH_4) using H_2 or formate as electron donor (Carbonero *et al.*, 2012; Hedderich & Whitman, 2006).

CH_4 is a colorless, volatile inert gas that has long been thought to have no negative effects in humans other than discomfort from gaseous distension (Pimentel *et al.*, 2012). However, increasing evidence has linked CH_4 production to various disease states (Pimentel *et al.*, 2012). There has been a correlation observed between high breath CH_4 level (50ppm) and decreased intestinal motility (Pimentel *et al.*, 2006). Moreover, there is a strong association between methanogenesis and chronic constipation (Attaluri *et al.*, 2009; Pimentel *et al.*, 2012; Pimentel *et al.*, 2006).

2.2.2 Lactate and succinate

L-lactate and/or D-lactate are the major fermentation products of lactic acid bacteria, including lactobacilli, bifidobacteria, enterococci and streptococci and can also be produced by strict anaerobes such as *Eubacterium* spp. (Barcenilla *et al.*, 2000; Macfarlane, 1997). Under some environmental conditions succinate is a metabolic end-product of some *Bacteroidetes* like the genera *Alistipes* and *Bacteroides* (Macfarlane, 1997; Rautio *et al.*, 2003). Both lactate and

succinate are intermediates in the global fermentation process and are to varying extents metabolized to SCFA by cross-feeding species (Belenguer *et al.*, 2007; Bernalier *et al.*, 1999; Duncan *et al.*, 2004b; Macfarlane & Macfarlane, 2012).

Lactate tends to reduce residual pH more than SCFA (Rinttila & Apajalathi, 2013). However, it is rarely present in high quantities, as it is normally rapidly absorbed from the intestine or used as a substrate for lactate-utilizing bacteria, such as representatives of the genera *Eubacterium*, *Anaerostipes*, *Veillonella* and *Megasphaera* (Belenguer *et al.*, 2006; Duncan *et al.*, 2004a; Harmsen *et al.*, 2002b; Rinttila & Apajalathi, 2013) (Figure 6). Succinate is the intermediate for propionate formation through decarboxylation of this symmetrical compound in the succinate pathway (Hosseini *et al.*, 2011; Reichardt *et al.*, 2014) (Figure 6).

In chickens, different studies found that *Lactobacillus* spp. were dominant in the duodenum and ileum (Oakley *et al.*, 2014b; Stanley *et al.*, 2014). The presence of this species suggested the production of lactate but normally it's rapidly absorbed from the intestine or used by some butyrate-producing bacteria (Duncan *et al.*, 2004a). Also succinate is an intermediate that can be absorbed by the intestine. The study of Kimmich *et al.* showed that isolated chick intestinal epithelial cells take up succinate by a Na^+-coupled transport system (Kimmich *et al.*, 1991).

2.2.3 Short-chain fatty acids

SCFAs are the major end-products of bacterial metabolism (Macfarlane & Macfarlane, 2003). They are formed principally from polysaccharide, oligosaccharide, protein, peptide and glycoprotein precursors by anaerobic microorganisms. Carbohydrates are the most important SCFA progenitors (Cummings & Macfarlane, 1991). The rate and amount of SCFA production depends on the site of fermentation and the composition and density of the gut microbiota in combination with the type of (complex) dietary fibers available for microbial fermentation (Macfarlane *et al.*, 2006). SCFAs are rapidly absorbed from the gut lumen, but their subsequent distribution, fate and effects on host cell metabolism differs (Louis *et al.*, 2014).

The caecum in broiler chickens is the main fermentative chamber and, in comparison with other gastro-intestinal tract segments, contains the largest number of bacteria (Nabizadeh, 2012). Looking at the microbiota composition of the caecum showed us that the chickens have different bacterial groups who were able to produce SCFA in the caecum (Lu *et al.*, 2003; Oakley *et al.*, 2014b; Stanley *et al.*, 2014). Most of the characteristics of the SCFA in humans could extrapolate to the chickens.

2.2.3.1 Acetate

Acetate is the most abundant SCFA and is produced by most enteric bacteria as fermentation product (Louis *et al.*, 2014) (Figure 6). Acetogens are a group of obligate anaerobic bacteria related to the genera *Ruminococcus*, *Blautia*, *Clostridium* or *Streptococcus* (Drake *et al.*, 2008). These bacteria utilize the acetyl-CoA (Wood-Ljungdhal) pathway to synthesize acetate from CO_2 and H_2 or from formate (Bernalier *et al.*, 1996a; Bernalier *et al.*, 1996b; Doré *et al.*, 1995). This acetogenesis is the most prevalent pathway of H_2 utilization in the human colon (Rey *et al.*, 2010). Non-acetogenic anaerobes, which comprise most of the microbiota, dispose reducing equivalents by formation of other products in addition to or instead of acetate, including succinate, propionate, butyrate, formate, lactate and ethanol (Louis *et al.*, 2014). Less widely recognized is that acetate is also an intermediate that is consumed by the major butyrate-producing bacteria (Flint *et al.*, 2012a; Louis & Flint, 2009).

Acetate (75mM) enhances ileal motility (Scheppach, 1994). It is suggested that acetate may have a trophic effect on the colonic epithelium not only by local action, but also by increasing mucosal blood flow (Scheppach, 1994). So acetate, the principal SCFA in the colon, is readily absorbed and transported to the liver, and therefore less metabolized in the colon (Hijova & Chmelarova, 2007). As the liver takes up acetate, it is used as substrate for lipogenesis and gluconeogenesis (Tremaroli & Backhed, 2012). In human studies, acetate is often used to monitor colonic events because it is the main SCFA in the blood (Hijova & Chmelarova, 2007).

2.2.3.2 Propionate

Three different pathways are used by the human gut microbiota to produce propionate (Reichardt *et al.*, 2014) (Figure 6). It is mostly formed via the succinate pathway by *Bacteroidetes* and by some *Firmicutes* of the class *Negativicutes* (Louis *et al.*, 2014). The acrylate route from lactate is found in bacteria belonging to *Clostridium* cluster IX (Louis *et al.*, 2007; Reichardt *et al.*, 2014). A third pathway, the propanediol pathway, is employed by the butyrate-producing bacterium *Roseburia inulinivorans* with fucose as substrate (Scott *et al.*, 2006). The proportion of propionate that is present in total faecal SCFA correlates with the relative abundance of *Bacteroidetes*, which confirms that the succinate pathway is the dominant source for propionate production (Louis *et al.*, 2014; Salonen *et al.*, 2014).

Propionate of gut microbial origin is known to possess biological activities at the level of the intestinal epithelium, although it may affect other organs and tissues due to its efficient transport across the gut epithelium (Hosseini *et al.*, 2011). Propionate has potential health-promoting

effects that include anti-lipogenic, cholesterol-lowering, anti-inflammatory and anti-carcinogenic actions (Delzenne & Williams, 2002; Hosseini *et al.*, 2011; Jan *et al.*, 2002; Vinolo *et al.*, 2011). It inhibits stimuli-induced expression of adhesion molecules, chemokine production and consequently suppresses monocyte/macrophage and neutrophil recruitment, suggesting an anti-inflammatory action (Vinolo *et al.*, 2011).

2.2.3.3 Butyrate

Butyrate is produced by many *Firmicutes* species using either butyryl-CoA:acetate CoA-transferase or, less commonly, phosphotransbutyrylase and butyrate kinase to catalyze the final steps of the pathway (Duncan *et al.*, 2004a; Louis *et al.*, 2004) (Figure 6). The butyryl-CoA:acetate CoA-transferase route is used by several genera of the healthy gut microbiota including *Faecalibacterium*, *Roseburia*, *Anaerostipes* and *Butyricicoccus* (Eeckhaut *et al.*, 2011; Flint *et al.*, 2012a; Louis & Flint, 2009; Louis *et al.*, 2014; Louis *et al.*, 2010; Walker *et al.*, 2011).

Butyrate is preferentially used as energy source by colonocytes and plays a multifunctional role in intestinal cells (Hamer *et al.*, 2008). It influences a wide range of cellular functions that affect intestinal health.

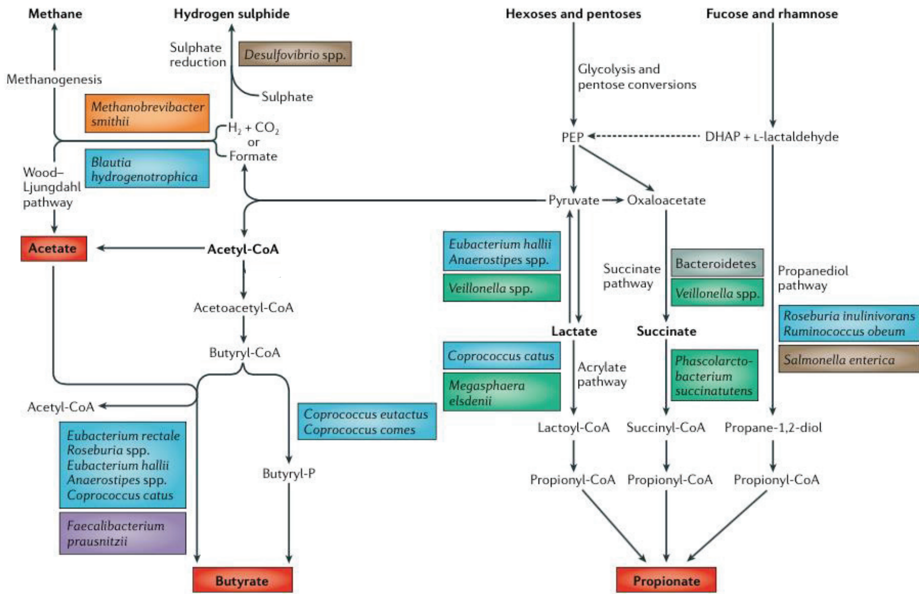

Figure 6. Pathways for the biosynthesis of the major microbial metabolites that result from carbohydrate fermentation and bacterial cross-feeding. Of the three main short-chain fatty acids (SCFAs; shown in red), acetate can be produced by many enteric bacteria from pyruvate via acetyl-CoA and also via the Wood-Ljungdahl pathway by acetogens, such as *Blautia hydrogenotrophica.* Butyrate is formed from two molecules of acetyl-CoA by several *Firmicutes*, and butyryl-CoA:acetate CoA-transferase is usually used to catalyze the last enzymatic step (Louis *et al.*, 2004). The predominant propionate production pathway is the succinate pathway, which is used by *Bacteroidetes* to generate propionate from carbohydrates and by some *Firmicutes* to produce propionate from lactate or succinate. Two other propionate formation pathways are found in some gut bacteria: the acrylate pathway, which uses lactate, and the propanediol pathway, which uses deoxyhexose sugars (such as fucose and rhamnose) (Reichardt *et al.*, 2014). The pathway that is involved in hydrogen metabolism is also shown. The bacterial species that are shown are based on studies of cultured isolates and metagenomic analyses and are thus not exhaustive. *Archaea* are shown in orange, *Bacteroidetes* are shown in grey, *Lachnospiraceae* (*Firmicutes*) are shown in blue, *Ruminococcaceae* (*Firmicutes*) are shown in purple, *Negativicutes* (*Firmicutes*) are shown in green and *Proteobacteria* are shown in brown. HAP, dihydroxyacetinephosphate; PEP, phosphoenolpyruvate (Louis *et al.*, 2014).

2.3 LIPID METABOLISM

A diet is composed of digestible and fermentable elements.. For carbohydrates and protein fermentation a lot of research has already been done on their microbial fermentation and the effects of the resulting metabolites, but for the lipid metabolism there is less research on this topic. Nevertheless it has been demonstrated that the microbiota acts at many levels, from lipid processing and absorption to systemic lipid metabolism and storage (Greer *et al.*, 2013; Tremaroli & Backhed, 2012).

The influence of dietary fat on the gut microbiota may be indirectly mediated by bile acids (Conlon & Bird, 2015). Bile acids are synthesized in hepatocytes as cholesterol moieties conjugated to either a taurine or a glycine amino acid and stored in the gallbladder before secretion into the duodenum via the common bile duct (Joyce *et al*., 2014; Brestoff & Artis, 2013). Bacterial bile salt hydrolase (BSH) enzymes in the gut catalyze the generation of unconjugated bile acids which are then further modified by bacteria to yield secondary bile acids. Functional BSH activity is a conserved microbial adaptation that is unique to the gut-associated microbiota and significantly influences lipid metabolism, weight gain and cholesterol levels in the host (Joyce *et al*., 2014).

However, further research is required on the interactions between dietary fat, the type and amount of bile acids that reach the large bowel, and the population structure and function of the microbiota (Conlon & Bird, 2015).

3. THE ROLE OF BUTYRATE IN INTESTINAL HEALTH

The four-carbon SCFA, butyrate, produced by microbial fermentation of dietary fibers, has multiple beneficial effects on host health (Canani *et al.*, 2011; Guilloteau *et al.*, 2010; Leonel & Alvarez-Leite, 2012). Butyrate is the energy source for epithelial cells in the hindgut and is involved in the maintenance of the gastro-intestinal mucosal health (Hamer *et al.*, 2008). In addition, butyrate stimulates the growth and differentiation of healthy epithelial cells but inhibits growth and induces apoptosis in human colon carcinoma cells and a variety of other tumor cell types (Comalada *et al.*, 2006; Luciano *et al.*, 2002).

3.1 ANTI-INFLAMMATORY FUNCTION

Butyrate exerts anti-inflammatory activity by several mechanisms, such as the inhibition of nuclear factor-kappa B (NF-κB) activation, interferon γ (IFNγ) production and the upregulation of peroxisome proliferator-activated receptor γ (PPARγ) (Inan *et al.*, 2000; Klampfer *et al.*, 2003; Wächtershäuser *et al.*, 2000) (Figure 7). NF-κB is a transcription factor that regulates genes involved in early immune and inflammatory responses (Baeuerle & Henkel, 1994). Butyrate is a histone deacetylase (HDAC) inhibitor. Hyperacetylation of histones has been reported to modulate the activity of the transcription factor NF-κB in a number of different cell types, including colon cancer cells and macrophages (Boffa *et al.*, 1978; Inan *et al.*, 2000; Luhrs *et al.*, 2002; Place *et al.*, 2005; Segain *et al.*, 2000). Inhibition of activation of NF-κB by butyrate results in decreased expression of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) (Place *et al.*, 2005; Segain *et al.*, 2000). The study of Place *et al.* demonstrated that butyrate influences NF-κB activity by preventing the proteasome-dependent degradation of ubiquitinated inhibitor-kappa B α (IκBα), which is an inhibitory protein that inhibits the translocation of NF-κB into the nucleus (Scherer *et al.*, 1995) (Figure 7A). This inhibition of ubiquitinated IκBα proteasome degradation appears to arise from butyrate's ability to directly inhibit HDAC activity, since similar results are obtained with the specific HDAC inhibitor trichostatin A (TSA) (Finco *et al.*, 1994; Miyamoto *et al.*, 1994; Place *et al.*, 2005).

The signaling of the pro-inflammatory cytokine IFNγ, secreted by lamina propria cells, is inhibited by butyrate at the level of phosphorylation of the signal transducer and activator of transcription 1 (STAT1) transcription factor (Klampfer *et al.*, 2003). The Janus kinases (JAK1/2) are responsible for the phosphorylation of STAT1. This results in STAT1 dimerization, its translocation to the nucleus, DNA binding and stimulated expression of the IFN-responsive genes (Jove, 2000; Schroder *et al.*, 2004). Klampfer *et al.* demonstrated that butyrate inhibits STAT1

activation at least in part through the inhibition of JAK2 phosphorylation, which suggests that JAK2 is the target of the butyrate action for the inhibition of IFNγ signaling (Figure 7B).

PPARγ is a ligand-activated transcription factor that belongs to the nuclear hormone receptor family and that participates in a variety of immune processes (Schwab *et al.*, 2007; Wächtershäuser *et al.*, 2000). In the human adenocarcinoma cell line Caco-2, butyrate increases PPARγ at mRNA and protein level (Wächtershäuser *et al.*, 2000). The PPARγ receptor is highly expressed in epithelium, indicating a role in the physiology of human colon cell proliferation and differentiation but also in modulating cell cycling (Desvergne & Wahli, 1999; Theocharis *et al.*, 2004). PPARγ can inhibit the action of NF-κB (Daynes & Jones, 2002; Dubuquoy *et al.*, 2002; Schwab *et al.*, 2007) (Figure 7C).

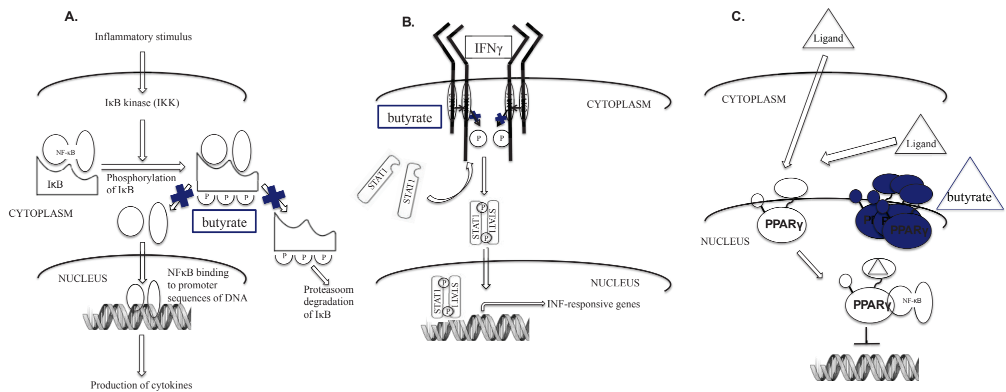

Figure 7. Butyrate exerts anti-inflammatory activity by several mechanisms. A. The inhibition of NF-κB. Inactive NF-κB dimers are sequestered in the cytoplasm of cells by the IκB family of inhibitory proteins. In response to inflammatory stimuli, IκB kinase is activated and IκB is phosphorylated, ubiquinated and degraded. The activated NF-κB complex translocates to the nucleus and binds DNA, regulating gene expression of the pro-inflammatory cytokines. Butyrate prevents the NF-κB activity by inhibiting the proteasome dependent degradation of ubiquitinated IκB. **B. The inhibition of IFNγ**. The binding of IFNγ to the IFN receptor initiates signaling through transphosphorylation and activation of the Janus kinases JAK1. This induces phosphorylation of STAT1, which results in STAT1 dimerization, its nuclear translocation, DNA binding and stimulation of expression of the IFN-responsive genes. The IFNγ-induced JAK2 activation is inhibited by butyrate. **C. The upregulation of PPARγ**. When a ligand binds to PPARγ, they prevent the activation of NF-κB. Butyrate increases PPARγ on mRNA and protein level and the activation of this receptor prevented NF-κB activity. NF-κB, nuclear factor-kappa B; IκB, inhibitor-kappa B; IFNγ, interferon-gamma; STAT1, signal transducer and activator of transcription 1; JAK1/2, Janus kinase1/2; PPARγ, peroxisome proliferator-activated receptor-gamma

3.2 STABILIZATION OF MUCOSAL BARRIER INTEGRITY

Butyrate also has a role in reinforcing the colonic defense barrier by affecting several components of this barrier (Canani *et al.*, 2011; Hamer *et al.*, 2008; Leonel & Alvarez-Leite, 2012). One of these components is the intestinal mucin 2 (MUC2) gene that encodes a glycosylated mucin (Jiang *et al.*, 2013). MUC2 mediated mucin production is stimulated by butyrate in human colon cancer cells, resulting in an important protective mucosal layer and thus protection against luminal agents (Hatayama *et al.*, 2007; Jiang *et al.*, 2013; Willemsen *et al.*, 2003).

Another important component are the tight junctions which form a major paracellular barrier in the epithelial tissues (Mitic *et al.*, 2000). The regulation of tight junctions depends on the activation of AMP-activated protein kinase (AMPK) (Zhang *et al.*, 2006). Butyrate increases the AMPK activity, which facilitates the assembly of tight junctions and thus enhances the intestinal barrier function (Peng *et al.*, 2009). The expression of Claudin-1, a major tight junction trans-membrane protein, is significantly upregulated by the addition of sodium butyrate (Wang *et al.*, 2012). The loss of intestinal epithelial integrity and will affect the protection against pathogens as well as uptake of nutrients and digestion.

The expression of antimicrobial peptides (AMP) such as cathelicidins and defensins forms a first line of defense protecting the gastro-intestinal mucosa against invasion and adherence of bacteria and thereby preventing infection (Hilchie *et al.*, 2013). In humans, there are various defensins while LL-37 is the only cathelicidin-derived peptide (Durr *et al.*, 2006). Butyrate increases the expression of LL-37 in a dose and time dependent manner by stimulating the activation of different mitogen-activated protein (MAP) kinases. The molecular mechanism may be linked to an increase in histone acetylation (Schauber *et al.*, 2003; Schauber *et al.*, 2004). In chickens, four cathelicidins (fowlicidins 1-3 and cathelicidin-B1) have been described (Goitsuka *et al.*, 2007; Xiao *et al.*, 2006). These cathelicidins are widely expressed throughout the GIT (Achanta *et al.*, 2012). Butyrate has a strong capacity to induce expression of AMPs such as defensins AvBD9 and AvBD14 and cathelicidin B1. Consequently, the supplementation of butyrate can augment disease resistance and reduce bacterial colonization in chickens (Sunkara *et al.*, 2014; Sunkara *et al.*, 2011).

3.3 PATHOGEN CONTROL

A correlation has been found between the presence of butyrate and the control of pathogens such as *Salmonella* Enteritidis and *Clostridium perfringens*. Different studies by Van Immerseel *et al.* show that butyrate significantly reduce *Salmonella* colonization and shedding in chickens and invasion of *Salmonella* in epithelial cell lines (Van Immerseel *et al.*, 2004a; Van Immerseel *et al.*, 2004b; Van Immerseel *et al.*, 2005). The invasion of intestinal epithelial cells is an important step in the pathogenesis of *Salmonella* infection and requires a set of genes encoded by the *Salmonella* pathogenicity island 1 (SPI1). Butyrate has been shown to suppress the HilA-dependent regulation of SPI1 by altering the regulation of *hilD* transcription (Gantois *et al.*, 2006). In addition to the effect on *Salmonella*, butyrate also has the ability to influence *C. perfringens*-induced necrotic enteritis (NE). NE is a widespread and economically important bacterial disease in broiler flocks that occurs in two forms (Van der Sluis, 2010). The subclinical form is characterized by poor performance (reduced growth, reduced feed efficiency) without mortality, while the clinical form appears with clinical signs and mortality (Shojadoost *et al.*, 2012). Butyrate has no significant antimicrobial effect against *C. perfringens*, but it is able to reduce the number of animals developing necrotic lesions in the small intestine in an *in vivo* model (Timbermont *et al.*, 2010).

3.4 GLUCAGON-LIKE PEPTIDE-2 UPREGULATION

How butyrate, which is mainly produced in the distal parts of the intestinal tract, can beneficially affect the small intestine is supposed to be due to the activity of glucagon-like peptide-2 (GLP-2) (Rowland & Brubaker, 2011). GLP-2 is a 33-amino acid peptide derived from the tissue-specific, post-translational processing of the proglucagon gene expressed in the intestinal enteroendocrine L-cells, which are located predominantly in the distal small intestine and colon (Burrin *et al.*, 2001; Holst, 2000). GLP-2 acts via specific binding with receptor GLP-2R, which is a G protein-coupled receptor (GPCR) (Drucker, 2001; Munroe *et al.*, 1999). The expression of GLP-2R is highly tissue specific and is located on enteric neurons, enteroendocrine cells (Guan *et al.*, 2006) and subepithelial myofibroblasts (de Heuvel *et al.*, 2012; Orskov *et al.*, 2005; Yusta *et al.*, 2000). These cells produce multiple downstream mediators affecting the intestinal epithelium (Drucker, 1999; Drucker & Yusta, 2014; Dube & Brubaker, 2007; Sigalet *et al.*, 2010). The different biological functions of the intestinotrophic peptide GLP-2 are related to the regulation of energy absorption and maintenance of mucosal morphology, function and integrity (Benjamin *et al.*, 2000; Drucker *et al.*, 1997; Drucker *et al.*, 1996; Estall & Drucker, 2006; Janssen *et al.*, 2013; Tsai *et al.*, 1997a; Tsai *et al.*, 1997b). A key beneficial effect of GLP-2 on the gut is its ability to increase intestinal epithelial growth by enhancing crypt cell proliferation and inhibiting apoptosis, resulting in increased villus height (Drucker *et al.*, 1996; Janssen *et al.*, 2013; Tsai *et al.*, 1997a; Tsai *et al.*, 1997b). These multiple functions display the benefit of GLP-2 in a setting of intestinal dysfunction (Dube & Brubaker, 2007). SCFA have been shown to mediate GLP-2 release by the enteroendocrine L-cells in the distal gut (Guilloteau *et al.*, 2010; Tappenden *et al.*, 2003). Butyrate appears to be the strongest stimulator of GLP-2 and is associated with increased plasma concentration of GLP-2 (Guilloteau *et al.*, 2010; Mangian & Tappenden, 2009; Tappenden *et al.*, 2003).

In chickens, GLP-2 is secreted by L-cells located in the epithelium of crypts and lower part of villi in the jejunum and ileum (Monir *et al.*, 2014; Nishimura *et al.*, 2013). L-cells in the chicken are characterized by secretory granules, which store GLP-1 and GLP-2, and microvilli covering the apical surface (Nishimura *et al.*, 2013). Intraperitoneal injection of GLP-2 in broilers reverses the negative effects of corticosterone induced stress on morphology, absorptive functioning and weight of the small intestine and positively influences the feed conversion ratio (FCR), daily gain and final body weight (Hu *et al.*, 2010). On the other hand, intracerebroventricular administration of chicken GLP-2 potently suppresses feed intake. This suggests a role in the regulation of appetite and might function as an anorexigenic peptide in chicken brain (Honda *et al.*, 2014;

Honda *et al.*, 2015). GLP-1 is already known as an anorexigenic peptide. In animal models GLP-1 inhibits food and drink intake upon intracerebroventricular injection The study of Honda *et al.* raises the hypothesis that in chickens the mechanism underlying the anorexigenic action of GLP-1 is identical to that of GLP-2. This suggests that GLP-1 and GLP-2 might play an important role in the central regulation of appetite in chicks.

3.5 INFLUENCING PERFORMANCE

Different studies already evaluated the effect of unprotected or fat-coated butyrate and butyrate glycerides in chickens on performance and showed their ability to beneficially influence FCR and body weight gain (BWG), intestinal villi structure and carcass quality, suggesting that butyrate and its glycerides may serve as a possible alternative to antimicrobial growth promoters. This will not be further elaborated but just a small overview is given of the effect of different butyrate derivatives on the growth performance of broiler chickens (Moquet *et al.,* in preparation). In-feed supplementation of sodium butyrate results in an increased BWG, decreased FCR, and increased ratio of villus height to crypt depth (Hu & Guo, 2007). Chickens with a 0.4% or 0.6% unprotected butyrate treatment had significantly higher BWG and reduced FCR than the control when the whole experimental period of five weeks was considered (Panda *et al.*, 2009). While Zhang *et al.* showed no effect of unprotected butyrate supplementation on performance parameters (Zhang *et al.*, 2011). Fat-coated butyrate can significantly reduce FCR (Smulikowska *et al.*, 2009), increases feed intake and BWG (Hautekiet *et al.*, 2011). On the other hand, a mixture of butyrate glycerides (mono-, di- and triglycerides) was shown to have no significant effect on performance parameters, except a significantly higher slaughter weight (Aghazadeh & Taha Yazdi, 2012; Antongiovanni *et al.*, 2007; Leeson *et al.*, 2005; Mahdavi & Torki, 2009).

4. METHODS TO ENHANCE THE ENDOGENOUS PRODUCTION OF BUTYRATE

Butyrate provides a link between diet, intestinal microbiota and metabolic health (Brahe *et al.*, 2013). As already noted above butyrate has several beneficial effects on intestinal health. Because of those beneficial effects, it is a justifiable strategy to stimulate the butyrate production in the intestine. This can be done through the use of butyrogenic pro- and prebiotics.

4.1 PROBIOTICS

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) originally defined probiotics as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). At the end of 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP) organized a meeting to re-examine the concept of probiotics. The panel worded a more grammatically correct definition and support use of this wording going forward. The definition is inclusive of a broad range of microbes and applications, whilst capturing the essence of probiotics (microbial, viable and beneficial to health). The distinction between commensal microorganisms and probiotics is also inferred from this definition (Hill *et al.*, 2014). The definition of probiotics is from now on "Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill *et al.*, 2014).

A probiotic can act in a number of ways. It can interact with the gut microbiota or it can exert its effect by bacterial enzymatic activities (Rijkers *et al.*, 2010). More-over, a probiotic can exert beneficial effects by interacting with the gut mucus and the epithelium, exert effects on the gut barrier, modify digestive processes, and stimulate the mucosal immune system and the enteric nervous system. Finally, probiotics can have beneficial effects outside the GIT, on the liver, the systemic immune system, and other target organs including the brain. Probiotics are not only able to influence the composition, but also the metabolic activity of the intestinal microbiota (De Preter *et al.*, 2011). It is important to note that each probiotic strain has its own specific properties (Gerritsen *et al.*, 2011). Most commonly used probiotic microorganisms belong to the bacterial genera *Lactobacillus* and *Bifidobacterium* (Boesten & de Vos, 2008; Kleerebezem & Vaughan, 2009). In recent years, however, there is also an increasing interest in the use of butyrate-producing bacteria as probiotics (Van Immerseel *et al.*, 2010).

4.1.1 Butyrate-producing bacteria as probiotic candidates

Combined efforts in species isolation (Barcenilla *et al.*, 2000) and metabolic characterization (Belenguer *et al.*, 2006) have led to the identification of functional groups of highly oxygen-sensitive micro-organisms within the phylum *Firmicutes* which are significant contributors to the butyrate pool. Most of the butyrate-producing bacteria have been shown to be related to clostridia, of which the full phylogenetic diversity has become apparent with the analysis of 16S rRNA gene sequences. In 1994, Collins *et al*. aligned the 16S rRNA sequences of more than 100 clostridial strains and assigned them to different clusters within the *Firmicutes* based on their phylogenetic interrelationship (Collins *et al.*, 1994). Recently, Yutin and Galperin proposed an updated taxonomic framework based on the phylogenetic reciprocal relation between different ribosomal proteins for the clostridia, in order to accommodate wrongly assigned species within the different clusters (Yutin & Galperin, 2013). This system, however, has not been widely adopted by the scientific community and so far, none of the proposed genus names has been validated by publication in the International journal of Systematic and Evolutionary Microbiology.

Butyrate-producing bacteria are an important component of the microbiota both in terms of abundance and functionality (Louis & Flint, 2009). The majority of the butyrate-producing bacteria are members of *Clostridium* cluster IV (e.g. *Faecalibacterium prausnitzii, Subdoligranulum variabile, Butyricicoccus pullicaecorum*, …) or XIVa (e.g. *Anaerostipes* spp., *Roseburia* spp., *Eubacterium hallii*, …), also referred to as *Clostridium leptum* group or *Ruminococcaceae* and *Clostridium coccoides* group or *Lachnospiraceae*, respectively. They constitute a major component of the total faecal human microbiota as well as the chicken caecal microbiota. Additional species responsible for colonic butyrate production are unevenly distributed and intermixed with non-butyrate producing genera within *Clostridium* cluster I (e.g. *Clostridium butyricum*), IX (e.g. *Megasphaera elsdenii*), XV (e.g. *Anaerofustis stercorihominis*) and XVI (*Eubacterium dolichum, E. cylindroides, E. biforme,* …) (Pryde *et al.*, 2002). It should be noted that most of these *Clostridium* clusters comprise a very heterogeneous assemblages of bacteria that do not form a phylogenetically coherent group. Therefore further studies are needed with respect to the classification of different species in these clusters. Chapter 1 of this thesis aims to contribute to this.

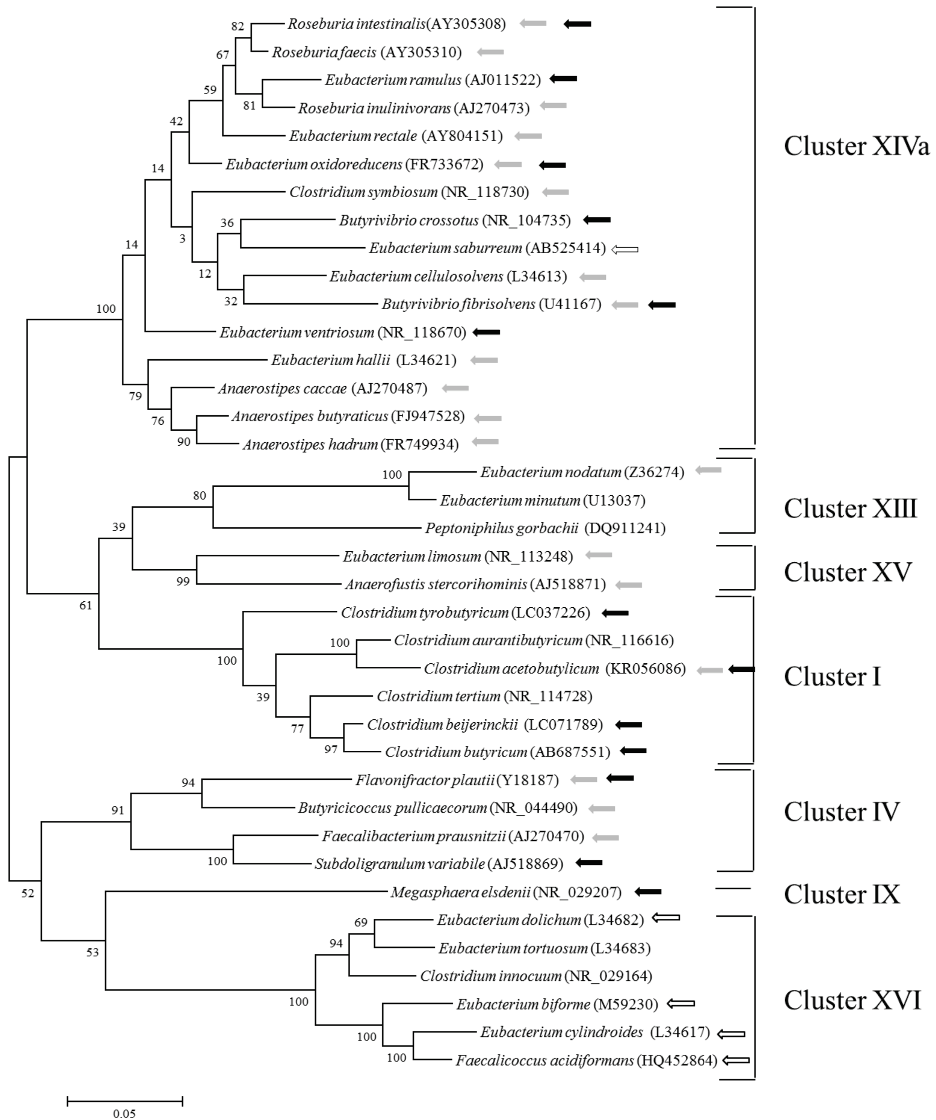

Figure 8: Maximum-likelihood phylogenetic tree of 16S rRNA gene sequences of butyrate-producing bacteria. Bootstrap values (expressed as percentages of 100 replicates) are shown at branch points. The scale bar represents genetic distance (5 substitutions per 100 nucleotides). The species are labelled with an arrow when possessing a sequence related to the butyryl-CoA: acetate CoA transferase gene (grey arrow), the butyrate kinase gene (black arrow) and the propionate-CoA transferase (transparent arrow). If no arrow is given then there was no information available in the literature.

It is clear that butyrate-producing bacteria are phylogenetically incoherent, are Gram-positive but can decolorize easily and appear Gram-negative or Gram variable, differ in DNA G+C content and also differ in their capacity to produce spores. Nevertheless, 16S rRNA and ribosomal

protein sequences have been shown to be better indicators of evolutionary proximity than phenotypic traits, when considering key phenotypical traits such as the structure of the cell envelope and Gram-staining pattern (Yutin & Galperin, 2013).

Butyrate-producers belonging to *Clostridium* cluster IV and XIVa have been shown to be significantly less abundant in the gastro-intestinal microbiota of human patients with IBD (Fava & Danese, 2011; Sartor, 2008; Sokol *et al.*, 2009). Frank *et al.* showed a correlation between reductions in abundance of the *Clostridium* cluster IV genera *Faecalibacterium* and *Butyricicoccus* and the IBD phenotype (Frank *et al.*, 2011). *F. prausnitzii* has anti-inflammatory effects by decreasing the synthesis of pro-inflammatory colonic cytokines and by inducing the secretion of anti-inflammatory cytokines (Sokol *et al.*, 2008). Different *in vivo* and *in vitro* studies support the probiotic potential of the species of this genus *Faecalibacterium*. *B. pullicaecorum* also seems to be a valuable probiotic candidate because of its anti-inflammatory potential in the IBD rat model (Eeckhaut *et al.*, 2013) and the good intrinsic bile acid tolerance (Geirnaert *et al.*, 2014). Despite the fact that *Clostridium* cluster XIVa is one of the most abundant groups of faecal bacteria in adult humans, there are currently no known members that comprise the properties of a probiotic (Hold *et al.*, 2002; Matsuki *et al.*, 2004). To our knowledge, the reason for this is not known. One possible reason is that *Clostridium* cluster XIVa is preferentially present in the mucus layer of the intestine and more difficult to isolate and to characterize as probiotic. *Clostridium* cluster XIVa can be divided into two sub-groups. The first subgroup is related to *Roseburia* and *Eubacterium rectale,* for which all available isolates show high levels of butyrate production, together with net acetate utilization (Aminov *et al.*, 2006; Barcenilla *et al.*, 2000; Louis & Flint, 2007; Pryde *et al.*, 2002). The second subgroup is related to *Eubacterium hallii* and *Anaerostipes caccae* and includes isolates that show the ability to convert acetate and D-/L-lactate into butyrate (Belenguer *et al.*, 2006; Duncan *et al.*, 2004a). Thus they can cross-feed on lactic acid produced by lactic acid bacteria, which co-occur in the gut. Stimulation of butyrate production may indeed explain the probiotic properties of some bifidobacteria and lactobacilli (Marteau, 2013; Veiga *et al.*, 2010).

Another butyrate-producing species that has documented probiotic potential in humans and animals is *Clostridium butyricum,* which belongs to *Clostridium* cluster I (Han *et al.*, 1984; Jonsson & Conway, 1992; Nakanishi *et al.*, 2003; Seki *et al.*, 2003). Administration of this bacterium has been shown to suppress chemically induced aberrant crypt foci and preneoplastic lesions in the rat colon (Araki *et al.*, 2000; Nakanishi *et al.*, 2003; Okamoto *et al.*, 2000). In addition, *C. butyricum* suppresses intestinal immune disorders by upregulating IL-10 production

(Hayashi *et al.*, 2013) and has beneficial effects on *Helicobacter pylori* infection in humans (Shimbo *et al.*, 2005). Together with *Bifidobacterium infantis*, it can restore microbiota changes and attenuate systemic inflammation in mice with antibiotic-associated diarrhea (Ling *et al.*, 2015).

Clostridium cluster XVI, also known as the *Erysipelotrichaceae* family, accounts for no more than 1% of the total faecal bacterial count in humans (Child *et al.*, 2006; Hold *et al.*, 2003) and harbors different butyrate-producing bacteria which produce considerably lower amounts of butyrate *in vitro* compared to the concentrations produced by butyrate-producing bacteria within *Clostridium* cluster IV and XIVa. At the time of writing, *Clostridium* cluster XVI comprises 14 different genera. The genus *Erysipelothrix*, for which 3 different species are described (Stackebrandt *et al.*, 2006), is facultative anaerobe with a weak fermentation activity. In addition to lactic acid, small amounts of acetic acid, formic acid and ethanol are produced (Reboli & Farrar, 1989). A second genus belonging to cluster XVI is *Holdemania*, isolated from human feces (Willems *et al.*, 1997). It is able to produce lactic and acetic acid as main fermentation end product in addition to small amounts of succinic acid from glucose. In 2000, four new genera were described and characterized. Based on the 16S rRNA sequences divergence different *Eubacterium*-like strains isolated from human faeces were classified within different new genera, for which the names *Coprobacillus, Catenibacterium* or *Solobacterium* were proposed ((Kagayama & Benno, 2000; Kageyama & Benno, 2000a, b). All species were shown to be obligatory anaerobic Gram-positive bacteria, able to produce acetic, lactic, butyric and iso-butyric acids from glucose fermentation ((Kagayama & Benno, 2000; Kageyama & Benno, 2000a, b). Species belonging to those genera may not be harmless, as several case reports have described *Solobacterium moorei* bacteremia in humans (Pedersen *et al.*, 2011; Lau *et al.*, 2006). The short bacilli isolated from human periodontal pockets and dento-alveolar infections were shown to be obligatory anaerobic, non-spore-forming, non-motile and Gram-positive and were classified within the genus *Bulleidia*, also within *Clostridium* cluster XVI (Downes *et al.*, 2000). Acetate, lactate and trace amounts of succinate are produced during glucose fermentation. From a blood culture of a febrile, 35-year-old man with acute appendicitis a bacterial species was isolated for which the genus name *Turicibacter* was proposed (Bosshard *et al.*, 2002). *Turicibacter is* anaerobic, Gram-positive, non-spore-forming, with lactate as main fermentation product. From the faeces of a dog, a *Eubacterium*-like species was isolated and classified to a new genus *Allobaculum*, based on the phenotypic and phylogenetic characteristics (Greetham *et al.*, 2004). This genus contains anaerobic, Gram-positive bacteria producing lactic and butyric

acid as end products of glucose metabolism. Phylogenetic analysis of the 16S rRNA sequences of *Lactobacillus catenaformis,* isolated from human faeces and intestinal and pleural infections and *Lactobacillus vitulinus*, isolated from bovine rumen contents, showed the relatedness of these two species with members of *Clostridium* cluster XVI (Salvetti *et al.*, 2011). Both species were reassigned to two new genera, *Eggerthia* and *Kandleria* respectively. They produce lactic acid as main fermentation product. From a stool sample of a patient suffering from anorexia nervosa, a strictly anaerobic species was isolated and named *Candidatus Stoquefichus* (Pfleiderer *et al.*, 2013). During a genomic update on clostridial phylogeny, misclassified clostridia such as *Clostridium ramosum* have been reclassified into the genus *Erysipelatoclostridium* (Yutin & Galperin, 2013). The genus *Dielma* is the only genus within *Clostridium* cluster XVI or family *Erysipelotrichaceae* that contains Gram-negative bacteria isolated from the human digestive tract, which are strictly anaerobic and motile. Nothing is yet described about their acid production after fermentation (Ramasamy *et al.*, 2013). Recently, a bacterium isolated from an anaerobic digester was classified within the new genus *Catenisphaera.* This genus is obligately anaerobic, Gram-positive, non-spore-forming, producing lactate as major end product and small amounts of butyrate, acetate, formate and hydrogen from glucose fermentation (Kanno *et al.*, 2015).

Besides the above mentioned genera there are still a few *Eubacterium*-like and *Clostridium*-like strains within *Clostridium* cluster XVI as well as a *Streptococcus sp.*. These species differ in shape, sporulation, fermentation end products and DNA G+C content from species of *Eubacterium, Clostridium* and *Streptococcus sensu stricto*. Therefore they need to be reclassified into new genera based on phylogenetic analysis of the 16S rRNA gene sequences. This task is partly accomplished in Chapter 1. In the study of Eeckhaut *et al*. concerning the diversity and phylogenetic relationship of cultivable butyrate-producing bacteria from the chicken caeca (Eeckhaut *et al.*, 2011), it was concluded that butyrate-producing bacteria related to *Clostridium* cluster XVI may play a more important role in the chicken gut than in the human colon. So far, not a single butyrate-producing strain within *Clostridium* cluster XVI has been tested or proposed as a candidate probiotic.

For the production of intracellular butyrate, two distinct pathways are described in clostridia (Gottschalk, 1986) (Figure 6). In the human gut only a few bacterial species use the butyrate kinase pathway with the intermediate formation of butyryl-phosphate (Louis *et al.*, 2004). The majority of the cultured human butyrate producing strains are found to carry the butyryl-CoA: acetate CoA-transferase gene that encodes the enzyme that transfers the CoA moiety to external acetate with the formation of acetyl-CoA and butyrate (Charrier *et al.*, 2006; Louis *et al.*, 2004;

Louis *et al.*, 2010). The butyryl-CoA:acetate CoA-transferase gene was detected in all *Clostridium* cluster XIVa and in the majority of the *Clostridium* cluster IV chicken strains. Only one *Clostridium* cluster IV chicken strain was shown to carry the butyrate kinase operon (Eeckhaut *et al.*, 2011). In all Clostridium cluster XVI isolates a CoA-transferase gene more closely related to propionate CoA-transferase was found. Analysis of draft genome sequences from cluster XVI butyrate producers revealed the presence of the gene directly downstream of the central pathway genes, which are involved in the formation of butyrate starting from acetyl-CoA. Therefore it was hypothesized that a CoA-transferase related to the encoding gene from *Clostridium propionicum* is responsible for butyrate formation in cluster XVI bacteria (Eeckhaut *et al.*, 2011).

The classification of bacteria is currently based on genetic and phenotypic information, which usually relies on growth in pure culture. Obtaining pure cultures is often time consuming and difficult and is especially challenging for microorganisms that have complex metabolic requirements (Yarza *et al.*, 2014). Especially for the interpretation of high-throughput sequence data from microbial communities, robust universal reference taxonomy is necessary. Taxonomy based on the 16S rRNA gene is the most comprehensive and widely used in microbiology today. However, two third of 16S sequences in GenBank are only classified to domain, that is Archaea or Bacteria, since most un(der)classified sequences are from culture-independent environmental surveys, which leaves users confounded about the phylogenetic affiliation of the submitted sequences (McDonald *et al.*, 2012). As such the classification and reclassification of misclassified clostridia and bacteria in general is important because of the implications for phylogenomic and metagenomic studies since different OTU classifiers use different taxonomic frameworks on which to base their sequence assignments.

In literature, there is not much published on these *Clostridium* cluster XVI bacteria and their characteristics. When looking at species belonging to this cluster, in the original article, Collins *et al.* makes an association of three species *Clostridium innocuum*, *Eubacterium biforme* and *Streptococcus pleomorphus* who were incoherent (Collins *et al.*, 1994). Looking at the phylogeny of these species, they were all three misnamed. The study of Eeckhaut *et al.* showed that four out of 6 isolates of cluster XVI from poultry were related to *Streptococcus pleomorphus*. Therefore it was decided to reclassify *Streptococcus pleomorphus*. The closest neighbours of this *S. pleormorphus* are *Eubacterium cylindroides* (94%) and *Eubacterium biforme* (93%). Both species were also misnamed, so it was decided to reclassify them also during the same study (Chapter 1).

Butyrate-producing bacteria may be considered to have health promoting effects on the host (Van Immerseel *et al.*, 2010). Unfortunately these bacteria are difficult to produce at large scale, because of the strictly anaerobic growth requirements and non-spore-forming characteristics. Therefore indirect stimulation to butyrate production in the gut, through the supply of carefully selected prebiotics or dietary fibres might be a valid alternative.

4.2 PREBIOTICS AND/OR DIETARY FIBRES

The concept of a prebiotic is relatively new, while some effects of fibres are already known for a long time (Gibson & Roberfroid, 1995; Gibson *et al.*, 2004; Roberfroid, 2007a; Slavin, 1987). Both improve intestinal health by different functions. The differences between prebiotic and dietary fibre are described below, both can be used as a feed supplement to improve the level of butyrate production in the intestine.

4.2.1 Prebiotics

A prebiotic was first defined as a "non-digestible ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves hosts health" (Gibson & Roberfroid, 1995; Gibson *et al.*, 1995). More specific criteria have been set in order to classify a food ingredient as a prebiotic (Gibson *et al.*, 2004). These criteria are (1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastro-intestinal absorption; (2) fermented by intestinal microbiota; and (3) selectively stimulating the growth and/or activity of those intestinal bacteria that contribute to health and well-being. The original definition of a prebiotic (Gibson & Roberfroid, 1995) only considers microbial changes in the colonic ecosystem. It may be good to extrapolate this to other areas of the host body that may benefit from a selective targeting of particular microorganisms (Gibson *et al.*, 2004; Roberfroid, 2007b). It has been proposed to refine the original definition of a prebiotic to "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastro-intestinal microbiota, that confer benefits upon host well-being and health" (Gibson *et al.*, 2004). Benefits of prebiotics include improvement of gut barrier function and host immunity, reduction of potentially pathogenic bacterial subpopulations (e.g. *C. perfringens*, *Salmonella*, *E. coli*, etc.) and enhancement of SCFA production (Slavin, 2013). Therefore prebiotics are considered alternative feed additives that can be used to improve poultry health and performance (Hajati & Rezaei, 2010).

4.2.2 Dietary fibres

According to the American Association of Cereal Chemists (AACC) report of 2001, the definition of dietary fibre is: "the edible parts of plants or analogous carbohydrates, that are produced during food processing by chemical and/or physical processes affecting the digestibility of starches or by purposeful synthesis, that are resistant to digestion and absorption in the human small intestine but completely or partially fermented in the large intestine. Dietary fibres include polysaccharides, oligosaccharides, lignin, and associated plant substances and promote beneficial

physiological effects including laxation, blood cholesterol and blood glucose attenuation" (DeVries, 2003). Dietary fibre is one of the most heterogeneous and diverse groups of molecules found in nature (Hamaker & Tuncil, 2014). According to the Codex Alimentarius (CAC/GL 2-1985, amendment: 2013) dietary fibres are defined as carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories: edible carbohydrate polymers naturally occurring in the food as consumed such as inulin, carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means like xylo-oligosaccharides (XOS) and synthetic carbohydrate polymers such as polydextrose which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities. Variation in their chemical structure affects their metabolization by the gut microbiota since bacteria have different abilities to cleave the linkages in the structure of these complex molecules to obtain simple sugars. The ability of gut microbes to utilize dietary fibre depends on their gene content, encoding carbohydrate-active enzymes (CAZymes) that are essential for cleavage of certain linkage types, and associated proteins such as carbohydrate-binding proteins and transporters (Hamaker & Tuncil, 2014). Fermentable fibres may provide a number of health benefits by altering the composition of the intestinal microbiota (Slavin, 2013). It is clear that not all fibres are equal in terms of the type and extent of health benefits they provide. Characteristics such as solubility, fermentability, and viscosity are important determinants for the effect the fibre will have in the body (Slavin, 2013). In general, dietary fibre represents the main source of energy for the bacteria of the GIT and accordingly, there exists the potential for its use to maintain or revert to a beneficial microbiota composition (Hamaker & Tuncil, 2014).

According to these definitions: 'All prebiotics are fibres, but not all fibres are prebiotics' (Slavin, 2013). Both, dietary fibre and prebiotics can be referred to as complex carbohydrates or non-starch polysaccharides (NSP). NSPs can be further subdivided based on chemical, physical and functional properties into soluble and insoluble fibres (Lattimer & Haub, 2010; Sizer & Whitney, 2008).

4.2.3 Soluble fibres

Soluble fibres dissolve in water, bypass digestion of the small intestine and are easily fermented in the large intestine (Hetland *et al.*, 2004; Lattimer & Haub, 2010).

4.2.3.1 Inulin and fructo-oligosaccharides

Inulin is a mixture of linear polymers and oligomers of fructose linked by a β-(2-1) glycosidic linkage often with a glucose terminal unit (Roberfroid *et al.*, 1998) (Figure 8). The number of monomeric units in a macromolecule or oligomer molecule is expressed as the degree of polymerization (DP) (Jenkins *et al.*, 1996). Inulin has a DP between 3 and 60 and is produced by extraction from foods like onions, garlic, wheat, artichokes and bananas by using a hot water diffusion process (Biedrzycka & Bielecka, 2004; Lattimer & Haub, 2010; Niness, 1999). Chemical degradation or controlled enzymatic hydrolysis of inulin using endoglycosidases results in oligofructose compounds with a DP between 2 and 20 (Roberfroid, 2007b; van de Wiele *et al.*, 2007). Fructo-oligosaccharides (FOS) and oligofructose are considered to be synonymous names for the mixture of small inulin oligomers with maximum DP of less than ten (Coussement, 1999; Gibson *et al.*, 2004; Quemener *et al.*, 1994). Oligofructose and FOS can be produced from sucrose by transfructosylation and from inulin by controlled hydrolysis (Niness, 1999). FOS usually contain between 2 and 4 β-(2-1)-linked fructosyl units (Rehman *et al.*, 2007; Roberfroid, 2002). The prebiotic effectiveness of inulin-type fructans not only depends on the dietary dosage, but also on the DP (van de Wiele *et al.*, 2007; Van Loo, 2004). The DP has an effect on the bifidogenic capacity of the fructans or in other words their specific capacity to stimulate bifidobacterial growth, on solubility, on the *in vitro* fermentation time and on the intestinal segment where the fermentation takes place (Harmsen *et al.*, 2002a; Roberfroid *et al.*, 1998; Tuohy *et al.*, 2001; Van Loo, 2004). This suggests that subtle differences in the chain length of carbohydrates can have a major impact on fermentation processes (Van Loo, 2004). A lower DP increases the bifidogenic capacity, leads to higher solubility and increases the speed of fermentation (Van Loo, 2004).

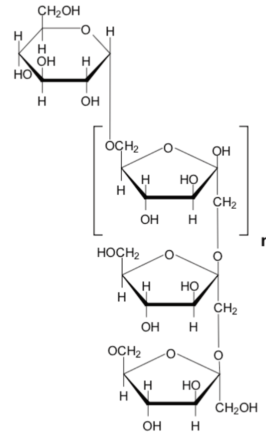

Figure 9. General chemical structure of inulin or inulin-like oligosaccharides (Braz de Oliveira *et al.*, 2011).

It is accepted that the main direct beneficial effect attributable to prebiotics is the induction of changes in the intestinal microbiota by selective stimulation of health-promoting bacteria (Gibson *et al.*, 1995). Fermentation studies in batch cultures using human faecal inocula show an increased number of bifidobacteria and low levels of *Escherichia coli* and *Clostridium* after supplementation with oligofructose and inulin (Wang & Gibson, 1993). Administration of chicory inulin hydrosylate to 8 healthy volunteers resulted in an increased number of faecal bifidobacteria (Menne *et al.*, 2000). In broilers, FOS has been shown to decrease the caecal concentration of *Campylobacter*, *Salmonella* (Yusrizal & Chen, 2003), *Clostridium perfringens* (Cao *et al.*, 2005; Kleessen *et al.*, 2003) and *E. coli* (Xu *et al.*, 2003; Yusrizal & Chen, 2003), and to increase bifidobacteria, lactobacilli (Xu *et al.*, 2003) and eubacteria levels (Cao *et al.*, 2005). In contradiction, other studies performed in broilers showed no effect of FOS supplementation (Fukata *et al.*, 1999; Rehman *et al.*, 2008; Rehman *et al.*, 2009; Yusrizal & Chen, 2003) or inulin supplementation (Biggs *et al.*, 2007; Yusrizal & Chen, 2003). In rats, FOS increases the numbers of caecal and faecal bifidobacteria (Hsu *et al.*, 2004; Koleva *et al.*, 2012). Inulin in broilers has a stimulatory effect on the growth of bifidobacteria and lactobacilli in the ileum (Rebole *et al.*, 2010) and a suppressive effect on the growth of *E. coli* in the caecum (Nabizadeh, 2012). The caecal *Lactobacillus* and *Bifidobacterium* populations were significantly increased in the inulin group compared with a control group of pigs (Tako *et al.*, 2008).

Conflicting results have been reported concerning the effect of inulin and FOS on growth performance. Both inulin and FOS gave an improvement in BWG when they were added to a

control diet in broilers (Nabizadeh, 2012; Rebole *et al.*, 2010; Xu *et al.*, 2003). This was in contrast with other studies that could not show an effect on performance for inulin (Alzueta *et al.*, 2010; Biggs *et al.*, 2007; Ortiz *et al.*, 2009; Rehman *et al.*, 2008) and FOS (Waldroup *et al.*, 1993; Williams *et al.*, 2008). In pigs, inulin had no effect on growth performance but it had an effect on digestibility and deposition of dietary minerals such as iron (Fe), calcium (Ca) and sulfur (S) (Jolliff & Mahan, 2012; Tako *et al.*, 2008; Yasuda *et al.*, 2006). The increase in tissue mineral content was accompanied by increases in liver and body weights in pigs (Jolliff & Mahan, 2012). Inulin and oligofructose increased caecum weight, decreased the luminal pH and increased the intestinal absorption of minerals such as Fe, Ca and Mg in rats (Coudray *et al.*, 2003; de Càssia Freitas *et al.*, 2012). The butyrate concentration was increased, the caecal pH was lower, and the relative caecal weight was higher when rats were fed FOS and oligofructose (Campbell *et al.*, 1997; Hsu *et al.*, 2004).

The apparently contradictory responses of inulin-type fructans may be explained by a variety of factors that can influence the outcome of these trials (Rebole *et al.*, 2010; Velasco *et al.*, 2010). These factors include inulin source, concentration, diet type, animal species, husbandry hygiene, animal age, and environmental stress conditions (Patterson & Burkholder, 2003; Verdonk *et al.*, 2005). Nevertheless, the results of different *in vitro* and *in vivo* experiments support the classification of inulin and oligofructose as prebiotics, since they fulfill the three above mentioned criteria (Gibson *et al.*, 2004).

4.2.3.2 Arabinoxylan and arabinoxylan-oligosaccharides

Arabinoxylan (AX) constitutes the major cell wall polysaccharide in cereal grains such as wheat, rye, barley, oats and other plants (Izydorczyk & Biliaderis, 1995). It consists of β-(1,4)-linked D-xylopyranosyl residues to which α-L-arabinofuranose units are linked as side chains (Cleemput *et al.*, 1993) (Figure 9). Some arabinofuranose units can be substituted with ferulic acid (Grootaert *et al.*, 2007). Arabinoxylan-oligosaccharides (AXOS) are oligosaccharides obtained by enzymatic or autohydrolytic depolymerisation of AX (Broekaert *et al.*, 2011). AXOS have different average degrees of polymerization (avDP, i.e. the average number of xylose residues in their backbone) and average degrees of substitution (avDS, i.e. the average ratio of arabinose to xylose), which may represent different prebiotic properties (Delcour *et al.*, 1999; Grootaert *et al.*, 2007; Sanchez *et al.*, 2009; Swennen *et al.*, 2005). The avDP of extracted material is calculated as the sum of the total arabinose and xylose contents, divided by reducing end xylose content (Van Craeyveld, 2009). Swennen *et al.* have described a large scale procedure for endoxylanase-mediated production of AXOS from wheat bran, yielding an AXOS preparation with an avDS of about

0.25-0.30 and an avDP ranging from about 3 to 15, depending on the enzyme mixture used (Swennen *et al.*, 2006).

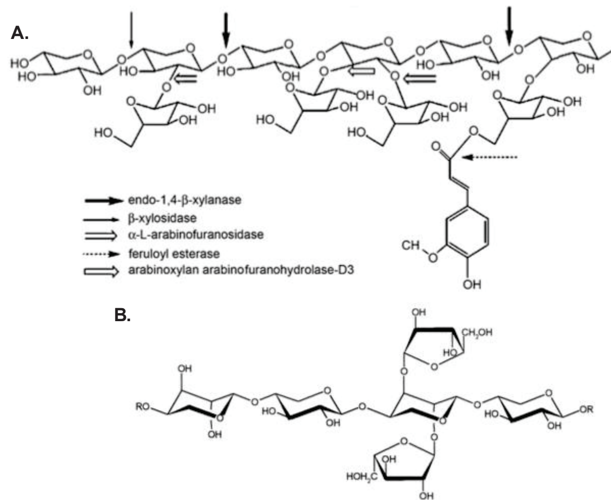

Figure 10 A. Chemical structure of AX and the site of activity of the different AX degrading enzymes (Grootaert *et al.*, 2007). **B.** AXOS from wheat bran hemicellulose (Vázquez *et al.*, 2000).

AXOS resist acidic hydrolysis due to the β-(1,4)-linkage (Courtin *et al.*, 2009; Okazaki *et al.*, 1991; Sanchez *et al.*, 2009). Humans appear to lack the enzymes able to degrade AX or AXOS and this suggests that AXOS remain largely unabsorbed in the small intestine (Broekaert *et al.*, 2011). In the human colon, the predominant endoxylanase-producing microbiota include species of the Gram-negative genus *Bacteroides* and of the Gram-positive genus *Roseburia,* which are necessary for degradation of AX (Broekaert *et al.*, 2011; Chassard *et al.*, 2007). AX from aleurone with low avDS were virtually completely degraded within 8h after inoculation with human faeces, whereas with AX from wheat bran with higher avDS, substantial amounts were still present after 24h (Amrein *et al.*, 2003). This indicates that AX fractions with high avDS are more difficult to degrade than AX with low or moderate avDS (Broekaert *et al.*, 2011; Karppinen *et al.*, 2001). Major fractions of AXOS were shown to be degraded within 24h after human faecal inoculation and complete *in vitro* fermentation was obtained after prolonged incubation for up to 80h with production of acetate, propionate and butyrate (Kabel *et al.*, 2002). The production of SCFA upon *in vitro* fermentation of AX and AXOS has been confirmed through *in vivo* studies (Broekaert *et al.*, 2011). For example, ingestion by human volunteers of an AXOS-rich preparation incorporated in bread raised acetate, propionate and butyrate levels (Grasten *et al.*,

2003). AXOS appear to be a relatively selective substrate. It can be utilized by some *Bifidobacterium* species and, though less efficiently, some *Bacteroides* and *Lactobacillus* species during *in vitro* fermentation (Van Laere *et al.*, 2000; Yamada *et al.*, 1993). *In vivo* intervention trials with rats, mice and chickens have also established that AXOS have strong bifidogenic properties (Broekaert *et al.*, 2011; Neyrinck *et al.*, 2012; Van Craeyveld *et al.*, 2008; Yamada *et al.*, 1993). In chickens, uncleaved AX is well known to hamper efficient feed utilization (Choct & Annison, 1992) and in-feed enzymes are used to cleave these polysaccharides. In addition, dietary AXOS provide dose-dependent protection against oral infections with *Salmonella* Enteritidis in poultry (Eeckhaut *et al.*, 2008).

AXOS derived from wheat bran have beneficial effects on feed utilization efficiency resulting in an improved FCR without increasing the body weight (BW) of broilers (Courtin *et al.*, 2008b). Feeding AXOS did not result in significant differences in growth in piglets, but it upregulated part of the small intestinal innate immune response (Niewold *et al.*, 2012). Colonic acetate and butyrate production increased and intestinal protein fermentation (branched SCFA and ammonium ion concentration) was reduced in rats fed a diet containing AXOS with a low avDP (≤ 5) but not with higher avDP (≥ 12) (Van Craeyveld *et al.*, 2008). AXOS administration did not affect the BW of rats (Van Craeyveld *et al.*, 2008) but had a protective effect on two types of preneoplastic lesions in a rat colon carcinogenesis model (Femia *et al.*, 2010). In mice, AXOS improved gut barrier function by looking at the tight junctions e.g. Claudin-3 protein was upregulated and decreased gut permeability by looking at the *zonula occludens* protein (Neyrinck *et al.*, 2012).

4.2.3.3 Xylo-oligosaccharides

Xylo-oligosaccharides (XOS) are sugar oligomers, which appear naturally in bamboo shoots, fruits, vegetables, milk and honey (Vázquez *et al.*, 2000). XOS are made up of a main backbone of xyloses linked by β (1→4) bonds, where the structural units are often substituted at position C2 or C3 with arabinofuranosyl, 4-*O*-methylglucuronic acid, acetyl, or phenolic substituents (Aachary & Prapulla, 2008; Ebringerová & Heinze, 2000) (Figure 10). The raw material for XOS synthesis are xylans extracted from lignocellulosic, xylan-containing materials like corncobs (Crittenden & Playne, 1996; Gullon *et al.*, 2008). Depending upon the various xylan sources used for XOS production, the structure of XOS can vary in DP (≤ 20), monomeric units and type of linkages (Aachary & Prapulla, 2011; Carvalho *et al.*, 2013). Xylan is hydrolyzed to XOS by the controlled activity of the enzyme endo-1,4-β-xylanase (Crittenden & Playne, 1996). Sometimes

also AX degradation as a results of activity of different enzymes such as endo-1,4-β-xylanases, α-L-arabinofuranosidases, β-xylosidases, α-glucuronidases or ferulic acid esterases, could give rise to XOS (Grootaert *et al.*, 2007) (Figure 9).

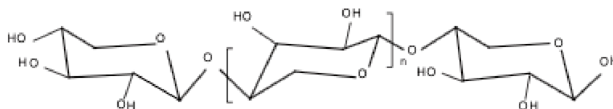

Figure 11. Schematic structure of xylo-oligosaccharides (Carvalho *et al.*, 2013).

XOS (DP=3) are more sensitive to alkaline decomposition than the longer chain AXOS (DP=15), but resist acidic hydrolysis under conditions representative for the gastric environment (Courtin *et al.*, 2009). The bifidogenic effect of XOS is observed in different *in vitro* fermentation studies and *in vivo* trials with humans, rats, mice, pigs and chickens (Crittenden *et al.*, 2002; Kabel *et al.*, 2002; Moura *et al.*, 2008; Okazaki *et al.*, 1990; Van Laere *et al.*, 2000). Many different *Bifidobacterium* species, even when grown in pure culture, can efficiently utilize XOS *in vitro* (Crittenden *et al.*, 2002). In addition, different *in vitro* studies show that only a small number of *Lactobacillus* species are able to efficiently utilize XOS as carbon source. In addition XOS are efficiently fermented by a limited number of dominant bacterial species such as *C. perfringens*, *E. coli*, *B. fragilis*, *B. vulgatus*, *Enterococcus faecalis* and *E. faecium* in the intestinal tract (Crittenden *et al.*, 2002; Moura *et al.*, 2008). A study of Scott *et al.* showed that XOS are a more selective growth substrate, because only 6 out of 11 *Firmicutes* strains are able to use XOS for growth (Scott *et al.*, 2014). The main fermentation products of XOS are acetate, lactate and/or butyrate (Kabel *et al.*, 2002).

In rats, XOS fermentation results in a decreased caecal pH and a lower serum triglyceride concentration, which reduces the risk of cardiovascular disease, but a higher total caecal weight and increased bifidobacteria population (Hokanson & Austin, 1996; Hsu *et al.*, 2004). XOS also increases caecal cell density via a modest enhancement of caecal epithelial cell proliferation in rats and mice (Howard *et al.*, 1995; Hsu *et al.*, 2004). XOS increases lactobacilli numbers by 10-fold and increases the counts of bifidobacteria in mice, while the levels of sulphite-reducing clostridia decrease significantly (Santos *et al.*, 2006). In humans, XOS also increases the *Bifidobacterium* counts but without a significant difference in the counts of *Lactobacillus*, *Enterobacteriaceae* and *Clostridium* (Finegold *et al.*, 2014; Na & Kim, 2007). XOS supplements in humans had no significant effect on stool pH, SCFA or lactic acid concentration (Finegold *et*

al., 2014). A study of Courtin *et al.* showed that the administration of XOS increased the number of bifidobacteria in the caeca of broilers relative to the control after only one week (Courtin *et al.*, 2008a). Zhenping *et al*. showed that broilers receiving a XOS-supplemented diet had a greater BWG and a decreased FCR than those receiving the non-supplemented diet (Zhenping *et al.*, 2013). To clearly understand the link between the beneficial effects of XOS on chicken performance and the composition and function of the microbiota, further studies are needed.

The results obtained in humans and animals show that AX, AXOS and XOS have beneficial nutritional effects. Nevertheless until today no study has classified them as prebiotics (Roberfroid, 2007b).

The choice to investigate the effects of XOS on broiler performance was based on the results of Zhenping *et al*. who showed that XOS had an effect on performance at slaughter age (Zhenping *et al.*, 2013). In this study, no analyses were performed to evaluate the effect of XOS on the microbiota composition although the *in vitro* study of Scott *et al*., had shown the ability of different butyrate producing bacteria to utilize XOS (Scott *et al.*, 2014). Since butyrate may be linked to improved performance a trial was set up in order to check what bacterial populations may be affected by XOS administration and may play a role in the improved performance (Chapter 2).

4.2.4 Insoluble fibres

Insoluble fibres do not dissolve in water, are indigestible and are generally less fermentable by colonic microbiota (Slavin *et al.*, 2009). However, they affect gut functioning and modulate nutrient digestion (Hetland *et al.*, 2004). Also in poultry, insoluble fibre is not degraded extensively by bacterial fermentation, which makes its influence on the composition and quantity of the microbiota relatively insignificant suggesting that insoluble fibres are inert and only act as nutrient diluents (Angkanaporn *et al.*, 2006; Choct *et al.*, 1996; Langhout, 1998).

The cell wall of the grain kernel is thick and hydrophobic. It consists of cellulose, xylans, and significant amounts of lignin. It primarily plays a role in protection of the kernel (Knudsen, 2015). Lignin is composed of highly cross-linked phenylpropanoid units like coniferyl, *p*-coumaryl, and sinapyl alcohols (Davin *et al.*, 2008; Lee, 1997) (Figure 11). Although lignin is not a carbohydrate, it is classified as one because of its tight association with cell wall polysaccharides (Knudsen, 2014). The microbial degradation of lignin has been studied in white-rot and brown-rot fungi, which use oxidative extracellular mechanisms to break down the lignin

polymer (Wong, 2009). Bacteria have been less well studied as degraders of lignin, but there are several reports of bacteria having this capacity (Bugg *et al.*, 2011; Taylor *et al.*, 2012; Zimmermann, 1990). Bacteria isolated from habitats such as soil containing lignified decaying plant material or from anaerobic ecosystems such as the rumen where lignocellulosic material is degraded, are expected to be able to metabolize lignin or lignin-derived compounds (Bugg *et al.*, 2011; Zimmermann, 1990). Bacterial strains identified to have lignin breakdown capacity fall into three classes: *Actinomycetes* (e.g. *Rhodococcus* sp., *Microbacterium* sp., *Streptomyces* sp.), α-*Proteobacteria* (e.g. *Paracoccus* spp., *Ochrobactrum* sp., *Shingomonas* sp.), and γ-*Proteobacteria* (e.g. *Pseudomonas* spp., *Acinetobacter* sp., *Citrobacter* sp.) (Bugg *et al.*, 2011).

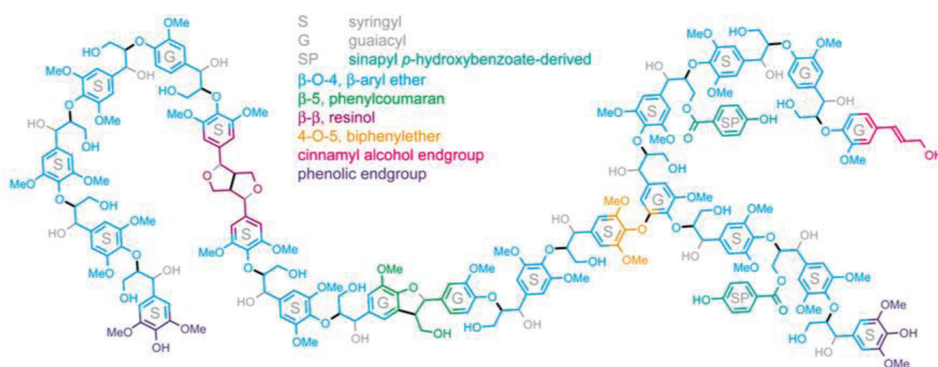

Figure 12. A lignin polymer from poplar, as predicted from NMR-based lignin analysis (Vanholme *et al.*, 2010)

4.2.4.1 Cellulose

Cellulose is composed of *β*-D-glucopyranose units linked by (1→4) glycosidic bonds and is an important structural component of cell walls in green plants and vegetables (Lattimer & Haub, 2010; O'Sullivan, 1997) (Figure 12). In the cereal co-product hulls, cellulose is the predominant polysaccharide, followed by xylans and pectin substances (Knudsen, 2014). Natural cellulose can be divided into two groups: crystalline and amorphous components (Lattimer & Haub, 2010). The group of crystalline components, which is made up of intra- and intermolecular non-covalent hydrogen bonds, is insoluble in water. The group of amorphous cellulose components have less intra- and intermolecular H_2 bonds, which make them more soluble (Ciolacu *et al.*, 2011). However, many modified celluloses such as powdered cellulose, microcrystalline cellulose and hydroxypropylmethyl cellulose have been developed and are used as food ingredients (Lattimer & Haub, 2010; Takahashi *et al.*, 2003). These modified celluloses differ from the natural

cellulose by the extent of crystallization and H_2 bonding whereby they are more soluble (Lattimer & Haub, 2010; Takahashi *et al.*, 2003). Wood shavings which are used in many countries as litter material consist of cellulose-rich crystalline fibre structures with great mechanical strength, determined by H_2 bonding density and conformation (Hetland *et al.*, 2004). However, their effects on digestibility, gut functions and bird behavior are largely unknown, when they are eaten (Hetland *et al.*, 2004). Cellulose is notoriously difficult to hydrolyze enzymatically because it contains resilient glycosidic bonds and is tightly associated with other polysaccharides (Mba Medie *et al.*, 2012). Two main types of cellulolytic enzyme activities are well characterized. Endoglucanases hydrolyze internal bonds at random positions of less ordered (or amorphous) regions of cellulose and these enzymes generate chain ends for the action of the second types of cellulases, the cellobiohydrolases (Mba Medie *et al.*, 2012). These are exoglucanases, which act in a unidirectional manner from the ends of cellulose polysaccharide chains and liberating cellobiose as the major product (Mba Medie *et al.*, 2012).

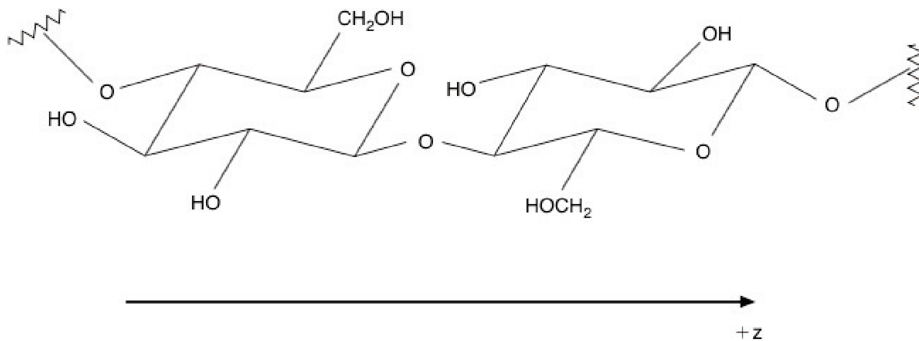

Figure 13: Fragment (repeating unit) of a cellulose chain (O'Sullivan, 1997).

Cellulose is considered to be a non-fermentable fibre, although it can go through microbial fermentation to a certain degree in the large intestine resulting in the production of SCFA (Chen *et al.*, 2015; Lattimer & Haub, 2010). The digestion of cellulose is therefore largely restricted to a specific group of cellulolytic microorganisms that produce a complex combination of enzymes (cellulases, hemicellulases and pectinases) that act synergistically to break down cellulose (Leschine, 1995; Mba Medie *et al.*, 2012). Cellulose-degrading species isolated from human faeces are *Clostridium* spp., *Eubacterium* spp., *Enterococcus* spp., *Ruminococcus* spp. and *Bacteroides* spp. (Betian *et al.*, 1977; Hamaker & Tuncil, 2014; Montgomery, 1988; Robert & Bernalier-Donadille, 2003; Wedekind *et al.*, 1988). The end products of cellulose fermentation

are comparable between *Ruminococcus* and *Enterococcus*, with acetate and succinate being the main metabolites formed by all species of these genera (Robert & Bernalier-Donadille, 2003). In addition, there is also the production of a large amount of H_2 from cellulose fermentation, which is transferred between H_2-producing fibrolytic species, acetogenic or sulphate-reducing bacteria and methanogenic archaea. This suggests that cellulose-degrading bacteria could play a role in the establishment and the development of methanogens in the gut (Robert & Bernalier-Donadille, 2003). Another study showed that the structure of the cellulolytic community varies depending on the presence of methanogens in the human gut (Chassard *et al.*, 2010). They showed that the main cellulose-degrading bacteria belong essentially to *Bacteroidetes* in non-methane-excreting subjects, while they are predominantly represented by *Firmicutes* in methane-excreting individuals (Chassard *et al.*, 2010).

The effects of the microcrystalline cellulose diet in rats are unclear (Campbell *et al.*, 1997). In young adult male rats 10 % cellulose showed no effect on food intake compared with the control group (Adam *et al.*, 2014). A study with male piglets showed that microcrystalline cellulose alone does not affect intestinal barrier function and it also has no significant effect on *Bacteroidetes*, Lactobacilli, *Enterobacteriaceae* and *Bifidobacterium* in the caecum compared with the control or AXOS group (Chen *et al.*, 2015). Although powdered cellulose is regarded as a nonfermentable fibre, it can be degraded *in vitro* by cellulolytic bacteria of faecal inocula derived from various animal species (Johathan *et al.*, 2012).

Many studies have evaluated the effect of cellulose in different models, but the data are contradictory and this may depend on type of cellulose used in those models and other unknown factors (Lattimer & Haub, 2010). For example natural cellulose has been shown to decrease the postprandial glucose and insulin levels in rat, dog and cat but has no effect in pigs and humans (Lattimer & Haub, 2010; Nelson *et al.*, 1991; Nelson *et al.*, 2000; Nunes & Malmlof, 1992; Schwartz & Levine, 1980; Schwartz *et al.*, 1982). More consistent data are available when modified cellulose is used. Microcrystalline cellulose, methylcellulose and high viscosity hydroxylpropylmethyl cellulose decrease blood glucose levels in different hosts like pig, rat or human (Lattimer & Haub, 2010, Lightowler & Henry, 2009; Low *et al.*, 1985; Maki *et al.*, 2007; Takahashi *et al.*, 2005) suggesting that modified cellulose is more easily broken down by the host to glucose and absorbed in the intestine. Since the breakdown of natural cellulose to glucose takes longer, it passes the small intestine, making it for the host not possible to use the glucose as an energy source. Therefore modified celluloses such as powdered cellulose, microcrystalline cellulose and hydroxypropylmethyl cellulose may be more beneficial than natural celluloses. In

addition modified celluloses could act as soluble fibres and thus are able to improve the viscosity of the GIT (Lattimer & Haub, 2010). Most studies in poultry indicate that insoluble fibres like cellulose are inert and therefore act only as nutrient diluents (Hetland *et al.*, 2004). This was confirmed in a more recent study that determined the effect of purified fibre on the growth performance and intestinal health of young chicks (Wils-Plotz *et al.*, 2013). In this study powdered cellulose had no effect on the nutrient availability, which suggests that cellulose is essentially inert (Wils-Plotz *et al.*, 2013).

There is an increasing interest in the effects of cellulose on the host health because of the use as bulking agent to feed. More and more research is done in order to investigate the effect of the feed composition on the host health by evaluating the performance and microbiota composition. In order to correct the control medium for the added component in the test-medium, cellulose is added. Cellulose is seen as bulking agent, but is that correct? (Chapter 3)

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Scientific aims

The gastro-intestinal tract is the body site with the highest bacterial diversity and abundance, which is known to play a major role in metabolic, nutritional, physiological and immunological processes that influence health and disease (Gerritsen *et al.*, 2011; O'Hara & Shanahan, 2006). The microbial communities in the gut of livestock like chickens are becoming a focus of interest for veterinaries and animal nutritionists since they are believed to play an important role in host welfare and productivity (Chambers & Gong, 2011; Torok *et al.*, 2011). Although a lot of research has been done on the human intestinal microbiota, our knowledge of the chicken gut microbiota composition, metabolic function, and influence on animal health, welfare and performance is still incomplete. Understanding the dynamics of the gut microbial community or microbial balance is essential to establish or develop strategies to improve feed conversion efficiency and growth rate, to avoid intestinal diseases and proliferation of food-borne pathogens, and to identify feed additives and nutrients that support beneficial microbial communities. The metabolic activity of the intestinal microbiota results in the production of important metabolites such as short-chain fatty acids (SCFAs). Butyrate is considered one of the most important SCFAs, due to the multiple beneficial effects it has on human and animal health (Canani *et al.*, 2011; Guilloteau *et al.*, 2010; Leonel & Alvarez-Leite, 2012). The general aim of the present thesis was to further broaden our understanding of the role of prebiotics and microbial cross-feeding on butyrate production and butyrate producing microorganisms in broiler chickens. In the human gut, butyrate is mainly produced by species belonging to *Clostridium* cluster IV and XIVa (Collins *et al.*, 1994; Louis & Flint, 2009), however butyrate-producers related to C*lostridium* cluster XVI seem to play a more important role in the chicken gut than in the human colon (Eeckhaut *et al.*, 2011). Not many species of the *Clostridium* cluster XVI are yet described in detail and their function is still obscure.

Dietary carbohydrate intake has a major impact on the composition of the gut microbiota and its metabolic output (Duncan *et al.*, 2007; Flint *et al.*, 2012a; Flint *et al.*, 2012b; Scott *et al.*, 2014; Walker *et al.*, 2011; Wu *et al.*, 2011). Different prebiotics enhance health and performance by promoting the growth of specific beneficial groups of gut bacteria (Gibson *et al.*, 2004). They are known to influence *Bifidobacterium* and *Lactobacillus* species, but some also increase the level of butyrate-producing bacteria (Scott *et al.*, 2014). A far more detailed characterization of prebiotics or candidate prebiotics that influence the gut community in broilers is needed, especially with regard to the stimulation of butyrate-producing species.

Therefore, the specific aims of this work are:

- To characterize and reclassify different strains of the less abundant butyrate-producing *Clostridium* cluster XVI (Chapter 1).
- To analyse the effect of xylo-oligosaccharides (XOS) administration on the performance of broilers and to identify the XOS induced microbial shift in order to explain the beneficial effect on the gastro-intestinal health (Chapter 2).
- To analyse the effect of cellulose administration on the performance of broilers and their intestinal microbiota composition (Chapter 3).

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Chapter 1

Faecalicoccus acidiformans* gen.nov., sp.nov. isolated from the chicken caecum and reclassification of *Streptococcus pleomorphus*, *Eubacterium cylindroides* as *Faecalicoccus pleomorphus* comb.nov., *Holdemanella biformis* gen.nov., comb.nov. and *Faecalitalea cylindroides* gen.nov., comb.nov., respectively, within the family *Erysipelotrichaceae

Adapated from: De Maesschalck, C., Van Immerseel, F., Eeckhaut, V., De Baere, S., Cnockaert, M., Croubels, S., Haesebrouck, F., Ducatelle, R. and Vandamme, P. (2014) International Journal of Systematic and Evolutionary Microbiology 64, 3877-3884

ABSTRACT

Strains LMG 27428^T and LMG 27427 were isolated from the caecal content of a chicken and produced butyric, lactic and formic acid as major metabolic end products. The genomic DNA G+C content of strain LMG 27428^T was 40.4 mol% and 38.8 mol% for LMG 27427. On the basis of 16S rRNA gene sequence similarity, both strains were most closely related to the generically misclassified *Streptococcus pleomorphus* ATCC 29734^T. Strain LMG 27428^T could be distinguished from *S. pleomorphus* ATCC 29734^T based on higher lactic acid and less formic acid production in M2GSC medium, a higher DNA G+C content and absence of acid phosphatase, leucine, arginine, leucyl glycine, pyroglutamic acid, glycine and histidine arylamidase activity while strain LMG 27428 was biochemically indistinguishable from *S. pleomorphus*. The novel genus *Faecalicoccus* within the family *Erysipelotrichaceae* is proposed to accommodate strain LMG 27428^T (= DSM 26963^T) as *Faecalicoccus acidiformans* sp. nov. and strain LMG 27427 (= DSM 26962) as *Faecalicoccus pleomorphus* comb. nov.. Furthermore, the nearest phylogenetic neighbours of the genus *Faecalicoccus* are the generically misclassified *Eubacterium cylindroides* DSM 3983^T (94.4% 16S rRNA sequence similarity to the type strain) and *Eubacterium biforme* DSM 3989^T (92.7% 16S rRNA sequence similarity to the type strain). We present genotypic and phenotypic data that allow the differentiation of each of these taxa and formally propose to reclassify these generically misnamed *Eubacterium* species as *Faecalitalea cylindroides* comb. nov. (DSM 3983^T = ATCC 27803^T = JCM 10261^T) and *Holdemanella biformis* comb. nov. (DSM 3989^T = ATCC 27806^T = CCUG 28091^T), respectively.

INTRODUCTION

The complex microbiota of the gastro-intestinal tract is dominated by microorganisms belonging to the phylum *Firmicutes* (Ley *et al.*, 2008; Stanley *et al.*, 2013). Eeckhaut *et al.* investigated the diversity and phylogenetic relationships of butyrate-producing bacteria isolated from the chicken caeca and observed that butyrate producers belonging to *Clostridium* cluster XVI as determined by Collins *et al.* may play a more important role in the chicken gut than in the human colon (Collins *et al.*, 1994; Eeckhaut *et al.*, 2011). Members of *Clostridium* cluster XVI or the *Erysipelotrichaceae* family stain Gram-positive with incoherent cell morphology and include the generically misclassified *Streptococcus pleomorphus, Eubacterium cylindroides* and *Eubacterium biforme* (Collins *et al.*, 1994). The recent isolation of butyrate-producing bacteria from the caecal content of a 14-week old Isa Brown layer type pullet yielded strains LMG 27428^T and LMG 27427 (Eeckhaut *et al.*, 2011). They were isolated on M2GSC media at 38°C and in an anaerobic environment. Based on their near-entire 16S rRNA gene sequence, these strains appeared to be most closely related to the above-mentioned members of the *Erysipelotrichaceae* family. In the present study we describe the morphological, biochemical and genotypic characterization of strains LMG 27428^T and LMG 27427 and their nearest phylogenetic neighbours and propose a novel classification for each of these taxa.

METHODS

ISOLATION OF BUTYRATE-PRODUCING STRAINS

Strains LMG 27428^T and LMG 27427 were isolated from chicken caecal content and grown anaerobically for 48h on solid M2GSC medium pH 6 at 38°C (Eeckhaut *et al.*, 2011).

SEQUENCING AND PHYLOGENETIC POSITIONING

DNA was extracted from strains LMG 27428^T and LMG 27427 using an alkaline lysis procedure. Universal bacterial primers fD1 and rD1 (Weisburg *et al.*, 1991) and primers H279 and H280 (Goh *et al.*, 1996) were used to amplify the 16S rRNA gene and part of the 60kDa heat shock protein (*hsp60*) gene, respectively. After purification, the amplicons were sequenced by GATC Biotech (GATC Biotech AG, European Genome and Diagnostics Centre, Konstanz, Germany) using the same primers for *hsp60* and primers pD, Gamma*, 3 and O* for 16S rRNA (Coenye *et al.*, 1999). For the 16S rRNA sequence, the closest match to the deduced sequences was found using the EzTaxon-e server (Kim *et al.*, 2012), while for the *hsp60* gene sequence, an independent mapping against a reference *cpn60* gene database was used (Hill *et al.*, 2004). The sequences were aligned with reference 16S rRNA gene sequences and *hsp60* sequences using the MUSCLE program (Edgar, 2004a, b). Phylogenetic trees were constructed using MEGA 6 software (Tamura *et al.*, 2013; Tamura *et al.*, 2011). Clustering was determined with the maximum likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) and bootstrap values were calculated based on 100 replications.

DNA-DNA HYBRIDISATION

Genomic DNA of *S. pleomorphus* ATCC 29734^T and strain LMG 27427 was prepared according to (Pitcher *et al.*, 1989) and DNA-DNA hybridizations were performed as described by (Ezaki *et al.*, 1989) with an adapted hybridization temperature of 35°C.

DETERMINATION OF GENOMIC DNA G + C CONTENT

The mol% G+C content of strains LMG 27428^T and LMG 27427 was determined using a Waters Breeze HPLC system and an XBridge Shield RP18 column maintained at 37°C (Mesbah & Whitman, 1989).

QUANTIFICATION OF FATTY ACIDS METHYL ESTER COMPOSITION

The whole cell fatty acid methyl ester (FAME) composition was determined for strains LMG 27428^T, LMG 27427, ATCC 29734^T, DSM 3983^T and DSM 3989^T using an Agilent

Technologies 6890N gas chromatograph (Santa Clara, CA, USA). Fatty acids extraction and analysis of the fatty acid methyl esters were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI, Hewlett Packard, Newark, DE, USA). Fatty acids were extracted from cultures grown in M2GSC for 24h at 38°C under anaerobic conditions. The peaks of the profiles were identified using the TSBA50 identification library version 5.0 (MIDI, Hewlett Packard, Newark, DE, USA).

QUANTIFICATION OF SHORT-CHAIN FATTY ACIDS

The fermentation pattern of the two strains and their closest phylogenetic neighbours was analysed using HPLC-UV after grown in M2GCS for 24h at 38°C under anaerobic conditions (De Baere *et al.*, 2013).

RESULTS

Recently two strains LMG 27428^T and LMG 27427 were isolated and based on their near-entire 16S rRNA gene sequence, these strains appeared to be most closely related to three members of the *Erysipelotrichaceae* family namely *Streptococcus pleomorphus, Eubacterium cylindroides* and *Eubacterium biforme* (Collins *et al.*, 1994; Eeckhaut *et al.*, 2011). Both isolates were further characterized in detail and compared with their closest neighbours.

The colonies of both strains were 0.5-1.5mm in diameter and white in colour. The cell morphology was investigated using Gram-staining and scanning electron microscopy (SEM). Cells of both strains stained Gram-positive and were observed as cocci-bacilli-shaped pairs measuring 1.1-1.2µm and 0.9-1.0µm for strains LMG 27428^T and LMG 27427 respectively (Figure 1). Spore formation was not detected.

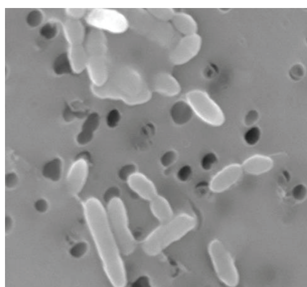

Figure 1. Scanning electron micrograph of cells of *Faecalicoccus acidiformans* LMG 27428^T.

The phylogenetic tree (Figure 2) showed that strain LMG 27428^T has moderate sequence similarity to *Streptococcus pleomorphus* ATCC 29734^T (96.0%) and lower similarity to *Eubacterium cylindroides* DSM 3983^T (94.4%) and *Eubacterium biforme* DSM 3989^T (92.7%). The 16S rRNA gene sequence of strain LMG 27427 was 99.6% similar to that of *S. pleomorphus* ATCC 29734^T. Phylogenetic analysis of protein encoding genes such as the *hsp60* gene is commonly used as an alternative identification instrument to distinguish between closely related species. The analysis shown in Figure 3 demonstrates that *S. pleomorphus* ATCC 29734^T and strain LMG 27427 have highly similar (98.2%) *hsp60* gene sequences that can be used to differentiate them from their nearest neighbour, i.e. strain LMG 27428^T. The level of DNA-DNA hybridization between strain LMG 27427 and *S. pleomorphus* ATCC 29734^T was 83% (the reciprocal hybridization values were 94 and 71%), which demonstrated that they indeed represent the same species.

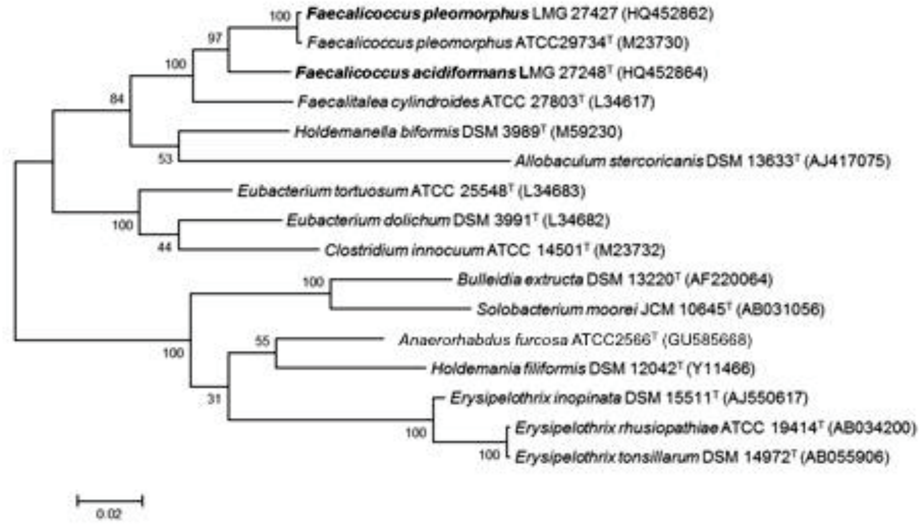

Figure 2. Rooted tree showing the 16S rRNA phylogenetic relationship of *Faecalicoccus pleomorphus* gen. nov., comb. nov. and *Faecalicoccus acidiformans* sp. nov. with some other members of the *Erysipelotrichaceae* family. The tree was constructed using the maximum likelihood method based on the Tamura-Nei model and based on a comparison of approximately 1300 nucleotides. Percentage bootstrap values, based on 100 replications, are shown at branch points. The accession numbers of reference organisms are included. Bar, 0.02 substitutions per nucleotide position. *Erysipelothrix rhusiopathiae* ATCC19414^T was used as outgroup. The evolutionary analyses were conducted using MEGA 6 software.

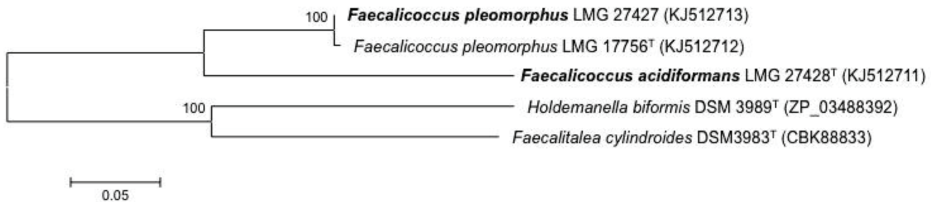

Figure 3. Phylogenetic tree based on the *hsp60* (heat-shock protein 60kDa) gene sequences of the two unknown and three reference strains. The *hsp60* gene sequence of two reference strains DSM 3989T and DSM 3983T was taken from the cpn60 database while for the two unknown strains and the reference strain LMG 17756T we sequenced them in this study. The tree, constructed using the maximum likelihood method based on the Tamura-Nei model, showed a comparison of two unknown and three reference strains of family Erysipelotrichaceae. Numbers at the nodes indicate the percentages of bootstrap sampling, derived from 100 samples, supporting the internal branches. The accession numbers of the peptide GenBank are included between brackets. Scale bar: 0.05 substitutions per nucleotide position. The evolutionary analyses were conducted using MEGA 6 software.

The genomic DNA G+C content of strains LMG 27428^T and LMG 27427 was determined to be 40.4 and 38.8 mol% respectively, which is similar to that of *S. pleomorphus* ATCC 29734^T (39.4 mol%) (Barnes *et al* 1977.).

Table 1: Cellular fatty acids profiles of two strains LMG 27428T and LMG 27427 and their closer phylogenetic neighbours of the family Erysipelotrichaceae (determined in this study). Strains: 1, *Faecalicoccus acidiformans* LMG 27428T; 2, *F. pleomorphus* LMG 27427; 3, *F. pleomorphus* ATCC 29734T; 4, *Faecalitalea cylindroides* ATCC 27803T; 5, *Holdemanella biformis* DSM 3989T. Major differences are highlighted in bold. ND, not detected

Cellular fatty acid	1	2	3	4	5
Saturated fatty acid					
$C_{10:0}$	0.9	0.7	0.6	1.2	1.0
$C_{11:0}$	0.8	0.2	ND	ND	ND
$C_{12:0}$	5.3	3.4	4.0	4.5	7.3
$C_{13:0}$	1.0	1.2	1.7	1.7	ND
$C_{14:0}$	7.5	6.3	9.0	9.0	4.4
$C_{16:0}$	**12.6**	**13.5**	**15.5**	**16.1**	**24.5**
$C_{18:0}$	4.6	7.1	10.6	7.0	**20.3**
$C_{19:0}$	ND	0.4	ND	ND	ND
Unsaturated fatty acids					
$C_{15:1}$ $\omega 6c$	ND	0.9	1.4	1.1	ND
$C_{16:1}$ $\omega 9c$	1.5	0.8	1.0	1.8	ND
$C_{17:1}$ $\omega 8c$	3.5	6.3	4.1	6.4	ND
$C_{17:1}$ $\omega 6c$	ND	1.3	0.8	1.0	ND
$C_{18:1}$ $\omega 9c$	**10.7**	**12.8**	**13.2**	**12.0**	**8.0**
$C_{18:1}$ $\omega 7c$	5.3	5.8	6.8	3.7	2.6
$C_{18:1}$ $\omega 6c$	ND	ND	ND	ND	3.5
$C_{20:1}$ $\omega 9c$	0.5	ND	ND	ND	ND
Branched fatty acids					
iso-$C_{14:0}$	ND	0.1	ND	ND	ND
iso-$C_{15:0}$	0.5	0.5	ND	ND	1.1
anteiso-$C_{15:0}$	1.0	0.7	1.1	0.8	2.5
iso-$C_{16:0}$	ND	0.3	ND	ND	0.9
iso-$C_{17:0}$	ND	0.4	ND	ND	ND
anteiso-$C_{17:0}$	ND	ND	ND	ND	1.3
iso-$C_{18:1}$ H	2.3	3.2	1.6	1.7	ND
iso-$C_{19:1}$ I	**17.3**	10.7	8.7	6.5	2.1
Hydroxyl fatty acids					
$C_{16:0}$ 3-OH	ND	0.4	0.6	ND	1.4
Summed features					
Summed feature 1	0.4	0.3	ND	1.1	ND
Summed feature 2	ND	0.7	ND	1.3	ND
Summed feature 3	4.4	5.4	6.0	7.3	0.4
Summed feature 4	11.2	9.6	7.0	9.0	8.7
Summed feature 5	2.6	2.3	2.1	2.0	7.1
Summed feature 6	1.1	1.9	1.3	0.7	ND
Summed feature 7	ND	0.2	ND	ND	ND

Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 1 = iso-$C_{15:0}$ H, $C_{13:0}$ 3-OH; Summed feature 2 = iso-$C_{16:1}$ I, $C_{14:0}$ 3-OH; Summed feature 3 = $C_{16:1}$ $\omega 7c$, iso-$C_{15:0}$ 2-OH; Summed feature 4 = iso-$C_{17:1}$, anteiso-$C_{17:1}$ B; Summed feature 5 = $C_{18:2}$ $\omega 6,9c$, anteiso-$C_{18:0}$; Summed feature 6 = $C_{19:1}\omega 11c$, $C_{19:1}\omega 9c$; Summed feature 7 = $C_{19:1}\omega 6c$, $C_{19:0}\omega 10c$.

The predominant fatty acid for strain LMG 27428^T was *iso*-$C_{19:1}$ (17.3%), while for the closest phylogenetic neighbours it was $C_{16:0}$ (13.5% - 24.5%) (Table 1). Also other fatty acids were present in lower percentages (above 1%) for the 5 strains: $C_{12:0}$ (3.4%- 7.3%), $C_{14:0}$ (4.4%- 9.0%), $C_{18:0}$ (4.6%- 20.3%), $C_{18:1}$ $\omega 9c$ (8.0%- 13.2%) and $C_{18:1}$ $\omega 7c$ (2.6%- 6.8%).

The strains LMG 27428^T, LMG 27427 and *S. pleomorphus* ATCC 29734^T produced 7 - 8.5mM lactic acid, 2.4 - 3.5mM butyric acid and 1.5-6.5mM formic acid (Table 2). Strains LMG 27428^T and LMG 27427 consumed 2.8-3.7mM acetic acid and 0.8-1.1mM propionic acid. Both *Eubacterium* strains DSM 3983^T and DSM 3989^T produced acids in the range of 0.3-4.0mM except for lactic acid of which the concentration was much higher for *E. cylindroides* DSM 3983^T (13.6mM).

Substrate utilization properties of strains LMG 27428^T and LMG 27427 were compared to those of their nearest phylogenetic neighbour species using the API 20 A, rapid ID 32A and API ZYM systems (bioMérieux) according to the manufacturer's instructions except that the incubation was performed anaerobically for API ZYM. *S. pleomorphus* ATCC 29734^T exhibited enzymatic activity for acid phosphatase and different arylamidases like leucine, arginine, leucyl glycine, pyroglutamic acid, glycine and histidine and differed from strain LMG 27427 only in alanine arylamidase and in gelatin hydrolysis. Strain LMG 27428^T fermented D-glucose and D-mannose, but not D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, D-xylose, L-arabinose, glycerol, D-cellobiose, D-melezitose, D-raffinose, D-sorbitol, L-rhamnose and D-trehalose. Hydrolysis of gelatin and aesculin was not detected. Strain LMG 27428^T did not exhibit arylamidase or acid phosphatase activity and could thus easily be distinguished from *S. pleomorphus* ATCC 29734^T. *E. cylindroides* DSM 3983^T only fermented D-saccharose and D-raffinose, while *E. biforme* DSM 3989^T fermented D-raffinose, D-mannitol, salicin, D-xylose and L-arabinose. Gelatin hydrolysis was observed for *E. biforme* DSM 3989^T, while *E. cylindroides* DSM 3983^T hydrolysed aesculin and exhibited esterase, ester lipase and α-glucosidase activity, hence allowing a straightforward differentiation of strain LMG 27428^T and its nearest phylogenetic neighbours (Table 2).

Table 2: Comparison between the two strains LMG 27428T and LMG 27427 and their closest phylogenetic neighbours of the family Erysipelotrichaceae. Strains: 1, *Faecalicoccus acidiformans* LMG 27428T; 2, *F. pleomorphus* LMG 27427; 3, *F. pleomorphus* LMG 17756T; 4, *Faecalitalea cylindroides* ATCC 27803T; 5, *Holdemanella biformis* DSM 3989T. All data are from this study unless indicated otherwise. +, Positive; -, Negative; ND, not detected.

Characteristic	1	2	3	4	5
DNA G+C content (%mol)	40.4	38.8	39.4*	31.0†	33.8‡
Fermentation acids (mM)					
Butyric acid	3.5 ± 0.2	2.4 ± 0.3	3.2 ± 0.3	2.6 ± 0.6	3.2 ± 0.8
Acetic acid	-3.7 ± 0.6	-2.8 ± 0.3	N.D	1.0 ± 0.3	2.4 ± 0.5
Propionic acid	-0.8 ± 0.1	-1.1 ± 0.1	N.D	1.3 ± 0.4	3.9 ± 0.2
Lactic acid	8.5 ± 0.2	7.9 ± 0.2	7.0 ± 0.3	13.6 ± 2.1	2.1 ± 0.4
Formic acid	1.5 ± 0.1	3.8 ± 0.1	6.5 ± 0.3	0.7 ± 0.2	0.3 ± 0.1
Acid production from (API system):					
D-Mannitol§	-	-	-	-	+
D-Saccharose§	-	-	-	+	-
Salicin§	-	-	-	-	+
D-Xylose§	-	-	-	-	+
L-Arabinose§	-	-	-	-	+
D-Raffinose§	-	-	-	+	+
Hydrolysis of:					
Gelatin§	-	+	-	-	+
Aesculin§	-	-	-	+	-
Production of (API system):					
Alkaline phosphatase‖	-	-	-	+	+
Esterase (C4)‖	-	-	-	+	-
Ester lipase (C8)‖	-	-	-	+	-
Acid phosphatase‖	-	+	+	+	+
α-Glucosidase‖¶	-	-	-	+	-
Arginine arylamidase¶	-	+	+	-	-
Leucyl glycine arylamidase¶	-	+	+	-	-
Leucine arylamidase‖¶	-	+	+	-	-
Pyroglutamic acid arylamidase¶	-	+	+	-	-
Alanine arylamidase¶	-	+	-	-	-
Glycine arylamidase¶	-	+	+	-	-
Histidine arylamidase¶	-	+	+	-	-

DNA G+C content data were taken from: *, (Barnes *et al.*, 1977); †,(Cato *et al.*, 1974); ‡, (Cato *et al.*, 1974) § api® 20A; ‖ api® ZYM; ¶ rapid ID 32A

DISCUSSION

The high degree of phenotypic similarity together with the DNA-DNA hybridisation value demonstrate that strain LMG 27427 should be classified within the generically misclassified *S. pleomorphus* ATCC 29734^T. In addition, strain LMG 27428^T is the nearest phylogenetic neighbour of the latter but can be distinguished from it by a considerable 16S rRNA divergence (4.0%), *hsp6*0 sequence analysis, a higher lactic acid production but lower formic acid production, a higher DNA G+C content and the absence of acid phosphatase and various arylamidases activities. *S. pleomorphus,* strain LMG 27428^T, and the generically misclassified *E. cylindroides* and *E. biforme* all belong to a single line of descent within the family *Erysipelotrichaceae* and can be distinguished by both genotypic and phenotypic characteristics. On the basis of these polyphasic taxonomic data we propose to classify strain LMG 27428^T as *Faecalicoccus acidiformans.* Fa.e.ca.li.coc'cus. N.L. adj. faecalis (from L.n. faex faecis), pertaining to feces; N.L. masc. n. coccus (from Gr. masc. n. kokkus, a grain, seed), a coccus; N.L. masc. n. Faecalicoccus, coccoid bacteria that are isolated from faecal material. A.ci.di.for'mans. N.L. n. acidum (from L. adj. acidus, sour), an acid; L. part. adj. formans, forming; N.L. part. adj. acidiformans, acid-forming bacteria. The type species is *Faecalicoccus acidiformans* and strain LMG 27428^T (= DSM 26963^T) is the type strain.

The generically misnamed *S. pleomorphus* (Kawamura *et al.*, 1995; Ludwig *et al.*, 1988) is reclassify as *Faecalicoccus pleomorphus*. ple.o.mor'phus. N.L. masc. adj. pleomorphus (from Gr. adj. pleos, full, and Gr. n. morphê, form, shape), pleomorphic, different forms for the bacteria. The type strain is LMG 17756^T (= ATCC 29734^T, DSM 20574^T).

Furthermore, there is a growing consensus that the genus *Eubacterium sensu stricto* should be restricted to the type species, *Eubacterium limosum* ATCC 8486^T, and its closest phylogenetic relatives, and that the majority of *Eubacterium* species therefore needs reclassification (Kageyama *et al.*, 1999; Moore *et al.*, 1976; Nakazawa & Hoshino, 1994; Willems & Collins, 1996). The considerable phylogenetic divergence between *E. biforme* DSM 3989^T*, E. cylindroides* DSM 3983^T and their nearest phylogenetic neighbours, and the difference in mol% G+C content, in lactic acid production and in various other biochemical characteristics, together warrant the reclassification of *E. biforme* and *E. cylindroides*, as *Holdemanella biformis* and *Faecalitalea cylindroides* respectively. Hol.de.man.el'la. N.L. fem. dim. n. Holdemanella, named in honor of Lillian V. Holdeman Moore, a contemporary American microbiologist, for her outstanding contribution to the bacteriology of anaerobes. Bi.for'mis L. fem. adj. biformis, two-

shaped, two-formed (pertaining to cellular morphology). The type species is *Holdemanella biformis* and strain DSM 3989^{T} (= ATCC 27806^{T}, CCUG 28091^{T}) is the type strain. Fa.e.ca.li.ta'le.a N.L. adj. faecalis (from L. n. faex faecis), pertaining to faeces; L.fem. n. talea, a rod; N.L. fem. n. Faecalitalea, rods isolated from faeces. Cy.lin.dro'i.des. Gr. n. kulindros, a cylinder; L. suff. –oides (from Gr. suff. eides, from Gr. N. eidos, which is seen, form, shape, figure), resembling, similar; N.L. fem. adj. cylindroides, cylinder-shaped. The type species is *Faecalitalea cylindroides* and strain DSM 3983^{T} (= ATCC 27803^{T}, JCM 10261^{T}) is the type strain.

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Chapter 2

The effects of xylo-oligosaccharides on performance and microbiota in broiler chickens

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ABSTRACT

In broiler chickens, feed additives, including prebiotics, are widely used to improve gut health and to stimulate performance. Xylo-oligosaccharides (XOS) are hydrolytic degradation products of arabinoxylans that can be fermented by the gut microbiota. In the current study it was aimed to analyze the prebiotic properties of XOS when added to the broiler diet. Administration of XOS to chickens, on top of a wheat/rye-based diet, significantly improved the feed conversion ratio. XOS significantly increased villus length in the ileum. It also significantly increased numbers of lactobacilli in the colon and *Clostridium* cluster XIVa in the caeca. Moreover, the number of gene copies encoding the key bacterial enzyme for butyrate production, butyryl-CoA:acetate CoA-transferase, was significantly increased in the caeca of chickens administered XOS. In this group of chickens, at species level, *Lactobacillus crispatus* and *Anaerostipes butyraticus* were significantly increased in abundance in the colon and caecum, respectively. *In vitro* fermentation of XOS revealed cross-feeding between *L. crispatus* and *A. butyraticus*. Lactate, produced by *L. crispatus* during XOS fermentation, was utilized by the butyrate-producing *Anaerostipes* species. These data show the beneficial effects of XOS on broiler performance when added to the feed, which potentially can be explained by stimulation of butyrate-producing bacteria through cross-feeding of lactate and subsequent effects of butyrate on gastro-intestinal function.

INTRODUCTION

Cereal fibers are composed of carbohydrate polymers that are resistant to digestion in the small intestine of monogastric animals but are completely or partially fermented in the distal gut, and are believed to stimulate gut health (McCleary, 2003). The main components of the cereal fiber fraction are arabinoxylans (AX), pectins, resistant starch, cellulose, β-glucans and lignin (James *et al.* 2003). Hydrolytic degradation of the heteropolymer AX results in a mixture of arabinose substituted xylo-oligosaccharides or arabinoxylan-oligosaccharides (AXOS) and non-substituted xylo-oligosaccharides (XOS) (Broekaert *et al.*, 2011). XOS are oligomers consisting of xylose units, linked through β-(1-4) linkages (Aachary & Prapulla 2008). Selective fermentation of XOS has been shown to induce changes in both the composition and activity of the gastro-intestinal microbiota, improving the health and well-being of the host. This suggests that XOS could fulfill the definition of a prebiotic (Gibson *et al.* 2004). The production of lactate and short chain fatty acids (SCFA), including butyrate, upon fermentation of XOS, has been confirmed in several *in vitro* and *in vivo* studies (Broekaert *et al.*, 2011; Scott *et al.*, 2014). Lactate can stimulate butyrate production due to cross-feeding between lactate-producing bacteria and lactate-utilizing butyrate-producing bacteria from *Clostridium* cluster XIVa (Duncan *et al.* 2004). Butyrate has proven beneficial effects on gastro-intestinal function, since it has anti-inflammatory properties, fuels epithelial cells and increases the intestinal epithelial integrity. In addition, butyrate has been shown to improve growth performance in production animals and to change the microbiota composition and metabolic activity of the microbial ecosystem in the intestine (Canani *et al.*, 2011; Guilloteau *et al.*, 2010).

Beneficial effects of XOS have already been described in rats. In these studies, XOS was shown to significantly increase the bifidobacteria and lactobacilli population in the caecum (Gobinath *et al.* 2010; Hsu *et al.*2004). An *in vitro* study using swine faecal microbiota showed the highest SCFA production during fermentation of XOS (Smiricky-Tjardes *et al.* 2003). To our knowledge, there is not much published research on the effect of XOS on the gastro-intestinal health. In broiler chickens a recent publication of Zhenping *et al.*, showed increased growth performance, enhanced endocrine metabolism and improved immune function after in feed supplementation of straw-derived XOS (Zhenping *et al.*, 2013). Moreover, XOS was shown to decrease the ileal lactic acid concentration and increase the caecal butyric and total volatile fatty acid concentration at 21 days of age (Graham *et al.*, 2003). The effect of XOS on the microbiota composition was only investigated in young chicks and showed an increase in the number of bifidobacteria after only 1 week of supplementation (Courtin *et al.*, 2008).

In the broiler chicken, the distal ileum, the caeca and the colon are regarded as fermentation chambers whose function is determined by the microbiota composition (Meimandipour *et al.*, 2009; Sekelja *et al.*, 2012). The chicken gut microbiota is dominated by species belonging to the phyla *Firmicutes* (up to 75%) and *Bacteroidetes* (between 10% and 50%) (Dumonceaux *et al.*, 2006; Gong *et al.*, 2002; Knarreborg *et al.*, 2002; Lepage *et al.*, 2013; Lu *et al.*, 2003; Qin *et al.*, 2010; Torok *et al.*, 2008). Around 90% of the bacteria in the chicken gastro-intestinal tract are unknown species, indicating that the knowledge of the intestinal microbiota of chickens is incomplete (Apajalahti *et al.*, 2004). The majority of sequences within the *Firmicutes* phylum belong to the families *Ruminococcaceae* and *Lachnospiraceae*, the so called *Clostridium* cluster IV and XIVa, respectively (Collins *et al.* 1994). Both families contain numerous members that are known to produce butyrate as a fermentation end-product and are therefore linked to beneficial effects on gastro-intestinal function (Duncan *et al.*, 2007; Pryde *et al.*, 2002). Whether the abundance of these groups in the distal gut of chickens is affected by XOS is unclear.

In the current study, we analyzed the effect of XOS administration on the performance of broilers. In addition, we aimed to identify the shifts in microbiota composition induced by XOS to explain possible beneficial effects on gastro-intestinal health, with emphasis on butyrate production.

Materials and Methodes

Additives/substrates

In the *in vivo* study corncob-derived XOS35 (Longlive Bio-technology, Shandong, China) was used as feed additive. XOS35 is a mixture of 35% XOS with a degree of polymerization (DP) between 2-7 and 65% maltodextrin. In the *in vitro* fermentation study XOS35, maltodextrin (Sigma-Aldrich, St. Louis, United States) and XOS95 (Longlive Bio-technology, Shandong, China), a mixture of 95% XOS with DP 2-7 and 5% xylose, were used. The XOS95 and maltodextrin were used be confirm that the effects of XOS35 in the in vivo trial were explained by the XOS.

Animals and diets

A total of 192 male and 192 female one-day-old Ross-308 broiler chickens were randomly divided in 12 pens (3 pens of female and 3 pens of male birds per treatment and 32 chickens per pen) and housed on solid floor covered with wood shavings. Light schedule was set to provide an 18h light/6h dark cycle. The infrared bulbs (1 per pen during the first week) together with the central heating system provided optimal temperature. All animals were fed a wheat/rye-based diet with XOS (experimental group) or without XOS (control group) of which the composition is shown in Table 1. Sunflower meal and rapeseed meal were used as protein source in the diet; those proteins are slower digested than others. By using these protein sources, the diet is a suboptimal diet compared with the commercially available ones. In this diet also rye was used in order to increase the level of non-starch polysaccharides (NSP). The experimental starter feed (fed from the first day of age until day 13) was supplemented with 0.2% XOS, the grower feed (fed from day 14 until day 26) and the finisher feed (fed from day 27 until day 39) were supplemented with 0.5% XOS. At 13, 26 and 39 days of age, all broilers and the feed leftovers were weighed per pen to calculate the feed conversion ratio (FCR), weight gain (WG) and feed intake (FI). At 26 days of age, three chickens per pen were euthanized by an intravenous overdose of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). The complete content of caecum and colon was collected and stored at -70°C, while a part of the ileum at the level of Meckel's diverticulum was fixed in 4% formaldehyde.

Table 1: The composition and nutrient content of the wheat/rye diet administered to chickens. Starter diet was given from day 1 until 13, grower was given from day 14 until 26 and finisher was given from day 27 until 39. **The treatment group received 0.25% XOS on the top during starter and 0.5% XOS during grower and finisher.**

	Starter diet	Grower diet	Finisher diet
Feedstuff (%)			
Wheat	44.00	46.51	49.78
Rye	5.00	5.00	5.00
Soybean meal (48)	23.30	19.78	16.61
Soybeans	7.50	5.00	5.00
Sunflower meal 27	2.50	2.50	2.50
Rapeseed meal	7.50	10.00	10.00
Animal fat	3.90	5.00	5.12
Soy oil	2.80	2.82	2.61
Vitamin + trace elements	1.00	1.00	1.00
$CaCO_3$	0.55	0.56	0.75
Di-Ca-phosphate	0.90	0.62	0.37
NaCl	0.21	0.21	0.19
Na-bicarbonate	0.10	0.10	0.10
L-Lys-HCl	0.14	0.15	0.20
DL-Methionine	0.50	0.70	0.70
L-Threonine	0.04	0.03	0.03
Phytase	0.02	0.02	0.02
Calculated nutrient composition (% as fed)			
Crude protein	23.00	21.50	20.50
Crude fat	10.23	10.66	10.91
Crude fibre	4.17	4.19	4.19
Non-soluble polysaccharides	13.87	13.98	13.83
Metabolisable energy (MJ/kg)	11.72	12.15	12.25
Starch	28.03	29.41	31.76
Lysine, digestible	1.12	1.03	1.00
Sulfur amino acids, digestible	1.10	1.27	1.25
Threonine, digestible	0.73	0.67	0.65
Valine, digestible	0.84	0.76	0.72
Ca	0.85	0.80	0.75
Available P	0.40	0.35	0.30
NaCl + KCl (mEq/kg)	247	225	208
Linoleic acid (18:2)	3.34	3.15	3.17

The difference between the calculated and measured (Weende analysis) value for protein was + 2.4% in starter and +7.2% in grower diet. For fat it was + 6.9% in starter and + 5.4% in grower diet.

MORPHOLOGICAL EXAMINATION

Formalin fixed ileum segments taken at the level of Meckel's diverticulum were dehydrated in xylene, embedded in paraffin and sectioned in 4µm slides. The sections were deparaffinized (2 x 5 min) in xylene, rehydrated in isopropylene (5 min), 95% alcohol (5 min) and 50% alcohol (5 min) and stained with haematoxylin and eosin. The sections were examined using light

microscopy. Villus length and thickness of *tunica muscularis* were measured by random measurement of 10 villi and 10 measurements of *tunica muscularis* per section using Leica DM LB2 Digital (Leica Microsystems Belgium BVBA, Diegem, Belgium) and a PC-based image analysis system, LAS V3.8 (Leica application Suit V3, Diegem Belgium).

MICROBIOTA COMPOSITION

DNA extraction

DNA was extracted from caecum and colon content using the CTAB method as described previously (Griffiths *et al.*, 2000; Kowalchuk *et al.*, 2000). To 100 mg of intestinal content, 0.5 g unwashed glass beads (Sigma-Aldrich, St. Louis, United States), 0.5 ml CTAB buffer (hexadecyltrimethylammonium bromide 5% (w/v), 0.35 M NaCl, 120 mM K_2HPO_4) and 0.5 ml phenol-chloroform-isoamyl alcohol mixture (25:24:1) (Sigma-Aldrich, St. Louis, United States) were added followed by homogenization in a 2 ml destruction tube. The samples were shaken 6 times for 30 seconds using a beadbeater (MagnaLyser, Roche, Basel, Switzerland) at 6000rpm with 30 seconds between shakings. After centrifugation (10min, 8000rpm), 300µl of the supernatant was transferred to a new tube. The rest of the tube content was re-extracted with 250µl CTAB buffer and again homogenized with a beadbeater. The samples were centrifuged for 10 minutes at 8000rpm and 300µl supernatant was added to the first 300 µl supernatant. The phenol was removed by adding an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma-Aldrich, St. Louis, United States) and a short spin. The aqueous phase was transferred to a new tube. The nucleic acids were precipitated with two volumes of PEG-6000 solution (polyethyleenglycol 30% (w/v), 1.6M NaCl) for two hours at room temperature. After centrifugation (20min, 13000rpm), the pellet was rinsed with one ml of ice-cold 70% (v/v) ethanol. The pellet was dried and resuspended in 100µl RNA free water (VWR, Leuven, Belgium).

Quantitative PCR for the total bacteria and the butyryl-CoA:acetate-CoA transferase gene

The number of total bacteria and butyryl-CoA:acetate-CoA transferase genes was quantified in 3 samples per pen (18 samples per treatment). To determine the number of total bacteria, primers Uni 331F (5'-TCCTACGGGAGGCAGCAGT-3') and Uni 797R (5'-GGACTAACCAGGGTATCTAATCCTGTT-3') were used (Hopkins *et al.*, 2005). Amplification and detection was performed using the CFX384 BioRad detection system (BioRad, Nazareth-Eke, Belgium). Each reaction was done in triplicate in a 12µl total reaction mixture

using 2x SensiMix™ SYBR No-ROX mix (Bioline, Kampenhout, Belgium), 0.5µM final primer concentration and 2µl of (50 ng/µl) DNA. The amplification program consisted of 1 cycle at 95°C for 10 min followed by 40 cycles of 1 min at 94°C, 1 min at 53°C and 2 min at 60°C. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was done after amplification and was obtained by slow heating from 60°C to 95°C at a rate of 0.5°C/5 sec to confirm the specificity of the reaction.

To quantify the number of gene copies encoding the butyryl-CoA:acetate-CoA transferase enzyme primers BCoATscrF (5'-GCIGAICATTTCACITGGAAYWS-3') and BCoATscrR (5'-CCTGCCTTTGCAATRTCIACRA ANGC-3') were used (Louis & Flint, 2007). Each reaction was done in triplicate in a 12 µl total reaction mixture using 2x SensiMix™ SYBR No-ROX mix (Bioline, Kampenhout, Belgium), 2.5µM final primer concentration and 2µl of (50 ng/µl) DNA. The amplification program consisted of 1 cycle at 95°C for 10 min followed by 40 cycles of 30 sec at 95°C, 30 sec at 53°C and 30 sec at 72°C.

16S rRNA gene sequencing to identify microbiota composition

Faecal samples derived from one animal per pen (6 per treatment) were used for 16S sequencing. For each sample, 16S rDNA PCR libraries were generated with the primers E9-29 and E514-430 (Brosius *et al.*, 1981) targeting hypervariable regions V1-V3. The oligonucleotide design included 454 Life Sciences's A or B sequencing titanium adapters (Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs) fused to the 5' end of each primer. The amplification mix contained 5U of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1x enzyme reaction buffer, 200µM dNTPs (Eurogentec, Liège, Belgium), 0.2µM of each primer and 100ng of genomic DNA in a volume of 100µl. Thermocycling conditions consisted of a denaturation at 94°C for 15 min followed by 25 cycles at 94°C for 40 s, 56°C for 40 sec, 72°C for 1 min and a final elongation step of 7 min at 72°C. These amplifications were performed on an Ep Master System gradient apparatus (Eppendorf, Hamburg, Germany). Electrophoresis of the PCR products was done on a 1% agarose gel and the DNA fragments were plugged out and purified using the SV PCR purification kit (Promega Benelux, Leiden, The Netherlands). The quality and quantity of the products were assessed with a Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium). All libraries were run in the same titanium pyrosequencing reaction using Roche MIDs. All amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche, Vilvoorde, Belgium), the sequence number of each sample is normalized to 2323 reads.

The 16S rDNA sequence reads were processed with the MOTHUR package (Schloss *et al.* 2009). The quality of all sequence reads were denoised using the Pyronoise algorithm implemented in MOTHUR and filtered with the following criteria: minimal length of 425bp, an exact match to the barcode and one mismatch allowed to the proximal primer. The sequences were evaluated for the presence of chimeric amplifications using Uchime (Edgar *et al.* 2011). The resulting read sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database 1.15 of full-length rDNA sequences (http://www.arb-silva.de/) implemented in MOTHUR (Pruesse *et al.* 2007). The final reads were clustered into OTUs using the nearest neighbour algorithm using MOTHUR with a 0.03 distance unit cut-off. At the OTU level of analysis (OTU definition level for a 0.02 distance matrix), a total of 3052 OTUs were created. A taxonomic identity was attributed to each OTU by comparison with the SILVA database (80% homogeneity cut-off). As a secondary analysis all unique sequences for each OTU were compared to the SILVA dataset 1.15 using BLASTN algorithm (Altschul *et al.* 1990). For each OTU, a consensus detailed taxonomic identification was given based upon the identity (less than 1% of mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not). The raw sequences were deposited in Genbank (accession number: PRJNA277118).

IN VITRO FERMENTATION

Bacterial strains, growth and co-culture studies

The butyrate-producing strain *Anaerostipes butyraticus* LMG 24724^T and the lactate producing strain *Lactobacillus crispatus* LMG 9479^T were purchased from the BCCM/LMG bacteria collection. *A. butyraticus* and *L. crispatus* were grown in M2GSC (Barcenilla *et al.*, 2000) and Man-Rogosa-Sharpe (MRS) medium, respectively, in an anaerobic chamber (Ruskinn technology, Bridgend, United Kingdom) with 84% N_2, 8% H_2 and 8% CO_2 at 37°C.

The *in vitro* fermentation study was conducted using a nutrient-poor medium described by Moura et al. (Moura *et al.*, 2007) with minor modifications (0.85 g/l casitone, 0.15 g/l enzymatic digest of soya bean, 0.25 g/l NaCl, 0.125 g/l K_2HPO_4, 5.0g/l bactopeptone, 5.0 g/l yeast nitrogen base and 0.5g/l resazurin. After autoclaving 1 mg/ml cysteine-HCl, 1% (v/v) of salt solution A (100.0 g/l NH_4Cl, 10.0 g/l $MgCl_2.6H_2O$, 10.0 g/l $CaCl_2.2H_2O$), 1% (v/v) trace solution (0.025 g/l $MnCl_2.4H_2O$, 0.02 g/l $FeSO_4.7H_2O$, 0.025 g/l $ZnCl_2$, 0.025 g/l $CuCl_2.2H_2O$, 0.05 g/l $CoCl_2.6H_2O$, 0.05 g/l SeO_2, 0.25 g/l $NiCl_2.6H_2O$, 0.25 g/l $Na_2MoO_4.2H_2O$, 0.314 g/l $NaVO_3$, 0.25 g/l H_3BO_3 dissolved in 0.02M HCl) and 1.2% (v/v) vitamin/phosphate solution (0.0204 g/l biotin, 0.0205 g/l

folic acid, 0.164 g/l Ca D-pentothenate, 0.164 g/l nicotinamide, 0.164 g/l riboflavin, 0.164 g/l thiamin HCl, 0.164 g/l pyridoxine HCl, 0.201 g/l *para*-amino benzoid acid, 0.0205 g/l cyanocobalamin dissolved in 54.7 g/l KH_2PO_4, filter sterile)) containing a mixture of SCFAs (final concentrations: acetate (31mM); propionate (9mM); isobutyrate, isovalerate and valerate (1mM each)). A 5% stock solution of XOS35, maltodextrin and XOS95 was prepared in the nutrient-poor medium, filter-sterilized (0.2µm), and diluted in the nutrient-poor medium to a final concentration of 0.5% (v/v). Un-supplemented nutrient-poor medium was used as control (blank). The final pH of the medium was adjusted to 6.5 ± 0.1. The media were pre-incubated in an anaerobic cabinet until anaerobiosis, as indicated by the colorless state of resazurin in the media. *A. butyraticus* and *L. crispatus,* pre-cultured in M2GSC and MRS broth, respectively, at 37°C under anaerobic conditions for 24 ± 1h without shaking, were diluted 100-fold in the supplemented and non-supplemented nutrient-poor medium. The co-culture of *A. butyraticus* and *L. crispatus* was prepared using equal portions of the inoculum (2 times 1/200) from the two pure cultures. After 24h anaerobic incubation at 37°C, bacterial growth was monitored by measuring the optical density at 650nm. After measuring the pH, the cultures were centrifuged at 14000rpm for 10 min at room temperature. The supernatants were stored at -20°C until lactate and butyrate concentrations were determined using high-performance liquid chromatography (HPLC) analysis. The *in vitro* fermentation assay was done twice in triplicate.

Determination of butyrate and lactate concentrations

DL-lactate and butyrate were quantified using HPLC with ultraviolet detection, as described by De Baere *et al.* (De Baere *et al.*, 2013). The supernatant was acidified using concentrated hydrochloric acid and extracted with diethyl ether for 20 min. The upper ether phase was transferred to another extraction tube and extracted again for 20 min with sodium hydroxide. The aqueous phase was transferred to an autosampler vial and concentrated hydrochloric acid was added. An aliquot was injected on the HPLC-UV instrument. The HPLC instrument consisted of a P1000X type quaternary gradient pump, an AS3000 type autosampler, an UV1000 type ultraviolet detector and a SN4000 type system controller, all from ThermoFisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved using a hypersilGold aQ column (150 x 4.6 mm, particle size: 3µm, ThermoFisher Scientific). Gradient elution (80/20) was performed using NaH_2PO_4 in HPLC grade water and HPLC grade acetonitrile as mobile phase A and B, respectively. The detector was set at a wavelength of 210nm. The Chromquest software (ThermoFisher Scientific) was used for data processing.

STATISTICAL ANALYSIS

The comparison of the performance data was performed with an independent samples t-test (SPSS 22.0). For the qPCR and morphology data were analyzed by means of a linear mixed effect model with pen included as random effect (S-Plus). The differences were considered statistically significant at P value ≤ 0.05 and considered as a tendency at P ≤ 0.1.

Statistical differences in bacterial population relative abundance between groups were assessed by non-parametric Kruskal-Wallis H test with Benjamin-Hochberg False Discovery Rate screen and Tukey-Kramer post-hoc test. Moreover, differences in specific bacterial population relative abundance based on 16S profiling were analyzed with non-parametric Mann-Whitney test using a two-tailed P value calculation.

GraphPad Prism software version 5 was used to perform the statistical analysis for the *in vitro* fermentation. All quantitative parameters (pH, OD, SCFA concentrations) were compared using the Kruskal-Wallis test. The Dunns *post hoc* test was applied for multicomparisons of these variables if there was a significant difference with the Kruskal-Wallis test.

RESULTS

BROILER PERFORMANCE AFTER SUPPLEMENTATION OF BROILER FEED WITH XOS35

To evaluate the effect of XOS on broiler performance, the body weight and feed intake were measured and FCR and growth were calculated. No significant differences were observed for body weight, feed intake or FCR parameters between the XOS-supplemented and non-supplemented group on day 13 (Table 2). The FCR was significantly (P = 0.01) better (lower) when chickens received the XOS-supplemented diet compared to the chickens receiving the control diet during the grower period (day 13 to day 26). When considering the starter and grower period together (day 0 to day 26), the FCR was significantly (P = 0.003) more favorable for chickens fed the XOS-supplemented diet compared to the chickens fed the control diet (Table 2). For the whole trial period (day 0 – 39), the FCR was also significantly improved (lower) for the group receiving the XOS-supplemented diet (P = 0.04). The average body weight at the different time points was non-significantly higher for chickens fed the diet supplemented with 0.5 % XOS compared to the chickens given the non-supplemented diet. These results together with the significantly improved FCR show a biologically relevant improved performance for chickens given the XOS-supplemented diet.

Table 2: The effect of XOS supplementation on the growth performance of the chickens. Feed conversion ratio (FCR), body weight (BW), feed intake (FI) and weight gain (Rey et al.) were measured at three time intervals for animals fed a wheat/rye-based diet without or with supplemented with 0.2% XOS (day 1-13) and 0.5% XOS (day 14-26; 27-39). Values are the mean of 6 pens with 32 chickens ± standard error of the mean. Statistical analysis was done with SPSS. Independent samples t-test was used to determine statistical differences between groups receiving non-supplemented and XOS supplemented diet. P-values less than 0.05 and 0.001 were considered significant (*, **).

Intervals in days		FCR	BW (g)	FI (g/d)	WG (g/d)
	-XOS	1.40 ± 0.05	331 ± 13.94	30.5 ± 0.83	22.0 ± 1.06
0-13	**+XOS**	1.38 ± 0.02	342 ± 8.02	31.4 ± 0.56	22.8 ± 0.63
	P-value	P = 0.76	P = 0.52	P = 0.36	P = 0.52
	-XOS	1.54 ± 0.02	1364 ± 15.39	104.9 ± 4.59	67.8 ± 2.26
13-26	**+XOS**	1.48 ± 0.01	1421 ± 16.97	108.1 ± 3.98	72.8 ± 2.28
	P-value	P = 0.01 (*)	P = 0.30	P = 0.54	P = 0.15
	-XOS	1.81 ± 0.01	2401 ± 60.01	165.6 ± 4.85	91.2 ± 2.72
26-39	**+XOS**	1.82 ± 0.01	2446 ± 57.26	162.2 ± 1.98	89.4 ± 0.73
	P-value	P = 0.59	P = 0.60	P = 0.54	P = 0.41
	-XOS	1.50 ± 0.01		67.3 ± 2.3	44,8 ± 1.50
0-26	**+ XOS**	1.45 ± 0.01		69.4 ± 2.04	47,8 ± 1.72
	P-value	P = 0.003 (**)		P = 0.50	P = 0.19
	-XOS	1.66 ± 0.01		100.0 ± 2.75	60.4 ± 1.53
0-39	**+ XOS**	1.63 ± 0.01		100.4 ± 2.43	61.6 ± 1.46
	P-value	P = 0.04 (*)		P = 0.93	P = 0.60

INTESTINAL MORPHOLOGY

Supplementation of 0.5% XOS to the broiler feed significantly (P = 0.04) increased the villus length in the ileum (Table 3). The *tunica muscularis* showed (P = 0.38) to be thicker in the group fed the XOS-supplemented diet (Table 3).

Table 3: The effects of XOS supplementation on the intestinal morphology of chickens on day 26. The data shown are the mean length of the villi (μm) and mean thickness of the tunica muscularis (μm) in ileal sections taken at day 26 of animals fed a wheat/rye-based diet, without or with supplemented with 0.5 % XOS (n=18). The length and the thickness were measured of 10 randomly selected villi and 10 different places for the tunica muscularis using a PC-based analysis system. Statistical analysis was done with S-plus, using a linear mixed effects model with pen as random factor. P-value less than 0.05 were considered significant.

	- 0.5% XOS	**+ 0.5% XOS**	
length of villi (μm)	1059 ± 40.00	1228 ± 59.79	P = 0.04
thickness of *tunica muscularis* (μm)	167.0 ± 11.01	178.9 ± 6.32	P = 0.38

MICROBIOTA COMPOSITION AS DETERMINED BY QPCR AND 16S SEQUENCING

There was no difference in the number of total bacteria between the XOS-supplemented and non-supplemented group in both the caecum and the colon (Figure 1A). The number of gene copies encoding the butyryl-CoA:acetate-CoA transferase was significantly (P = 0.02) higher in the caeca of the chickens that received 0.5% XOS (Figure 1B).

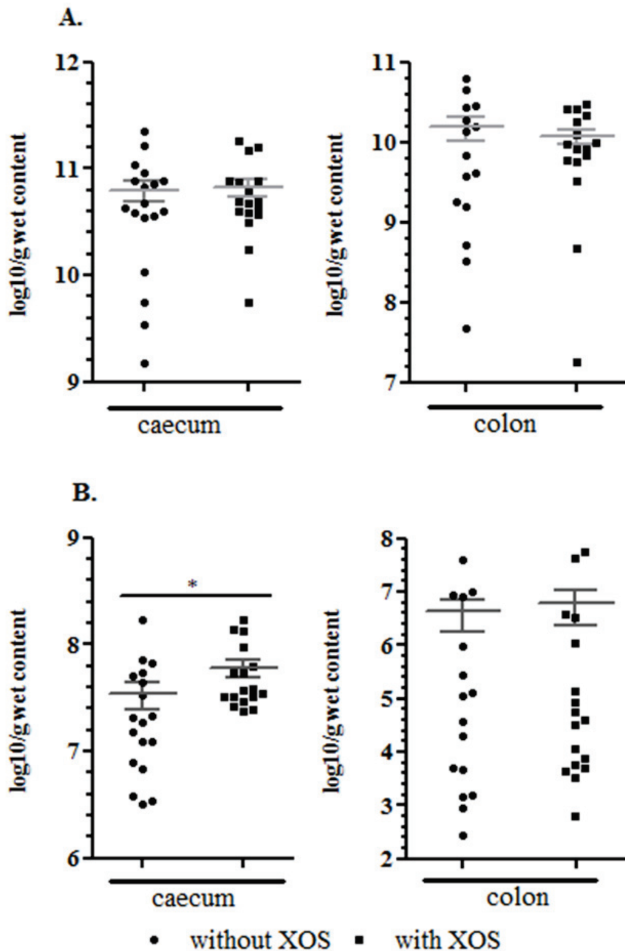

Figure 1. Number of total bacteria (A) and butyryl-CoA:acetate-CoA transferase gene copies (B) expressed as log10 copy number of the gene per g of wet content in the caecal and colonic content of 26-day old chickens fed a wheat/rye-based diet either or not supplemented with 0.5% XOS (18 chickens for each treatment). Statistical analysis is done with S-plus using a linear mixed effects model with pen as random factor to determine statistical difference between groups of animals fed a wheat/rye- based diet without and with XOS. * P ≤ 0.05

Significant changes were observed in the abundance of specific 16S sequences in caecum and colon samples at different taxonomic levels (Figure 2, Table 4). Supplementing XOS to the chicken diet resulted in a significant increase in the *Clostridium* cluster XIVa family in the caeca (P = 0.005, Figure 2). Chickens fed the XOS-supplemented diet showed a significant increase of unknown strains belonging to the butyrate-producing families *Clostridium* cluster IV and *Clostridium* cluster XIVa (Table 4). One of the species that also was found to be significantly more abundant in the caecum after supplementing XOS to the feed was *Anaerostipes butyraticus,* classified in the butyrate-producing *Clostridium* cluster XIVa (from 0.4% to 2.5%, P = 0.048, Figure 2). Supplementing XOS to the diet resulted in a significant increase in the *Lactobacillaceae* family (P = 0.033, Figure 2). At species level one species was found to be significantly more abundant in the colon after feeding the XOS-supplemented diet, *Lactobacillus crispatus* (from 4% to 15%, P = 0.007, Figure 2).

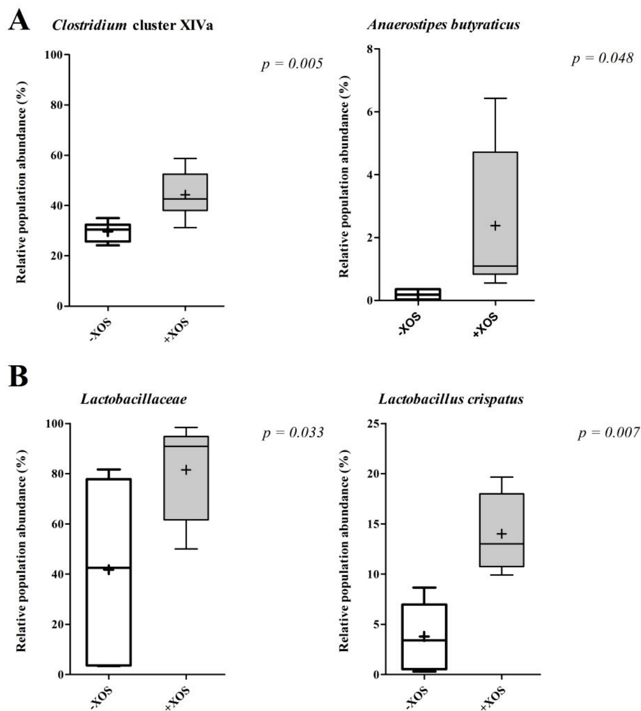

Figure 2. Box plots showing mean relative sequence abundance of the *Clostridium* cluster XIVa and *Anaerostipes butyraticus* in the caecum (**A**) and of the *Lactobacillaceae* and *Lactobacillus crispatus* in the colon (**B**) of 26-day old chickens fed without or with XOS-supplemented feed (6 chickens for each treatment). The plus represents the mean value and the whiskers are the median, the min/max value and 1ste/3rd quartiles.

Table 4: List of Clostridium cluster XIVa and cluster IV members identified in the caeca of chickens at day 26 of which the relative proportion was significantly different between the XOS supplemented and unsupplemented group. The results are based on the sequencing data of 6 chickens per treatment (ns, not significant).

				Highest 16S rRNA gene sequence similarity	
	- 0.5% XOS	+ 0.5% XOS	P-value	Type strain of validly named species (% 16S rRNA gene sequence similarity)	Accession number
***Clostridium* cluster XIVa**	29.64	44.29	0.004		
*Blautia*_RL199	0.04	0.15	0.04	*Blautia faecis* (95.96)	DQ793371
*Lachnospiraceae*_cc142	1.27	5.47	0.04	*Blautia schinkii* (93.93)	DQ057372
*Lachnospiraceae*_ic1296	0.34	0.79	0.03	*Blautia producta* (92.33)	DQ057459
*Lachnospiraceae*_GRC80	0.68	0.27	0.02	*Eubacterium contortum* (94.27)	DQ673545
*Lachnospiraceae*_B5-F3	1.19	7.28	0.01	*Blautia producta* (93.77)	EF025241
*Lachnospiraceae*_TS29	0.22	0.92	0.04	*Eubacterium hallii* (95.71)	FJ367509
***Clostridium* cluster IV**	29.12	29.31	ns		
*Ruminococcaceae*_BY13	0.69	0.05	0.04	*Pseudoflavonifactor capillosus* (96.23)	DQ342336
*Ruminococcaceae*_CFT19C1	0.00	0.08	0.04	*Clostridium alkalicellulosi* (84.4)	DQ455843
*Ruminococcaceae*_CFT212F12	0.05	0.16	0.01	*Oscillibacter valericigenes* (95.73)	DQ456381
*Ruminococcaceae*_RL246	0.19	0.78	0.02	*Clostridium alkalicellulosi* (85.44)	DQ793581
*Ruminococcaceae*_TS1	1.78	0.29	0.03	*Clostridium aldrichii* (85.66)	FJ365262
*Ruminococcaceae*_ELU0008	0.04	0.30	0.01	*Subdoligranulum variabile* (92.69)	HQ740050

IN VITRO FERMENTATION

To investigate cross-feeding between *L. crispatus* and *A. butyraticus* in the presence of XOS, an *in vitro* fermentation assay was carried out. XOS95 and maltodextrin were used to confirm the effect of XOS in the *in vivo* trial. Only the monoculture of *L. crispatus* resulted in a small pH drop when XOS35 was added to the medium (6.4 ± 0.04 versus 6.2 ± 0.04, Figure 3). *A. butyraticus* showed a significantly increased (P = 0.007) proliferation when XOS35 and XOS95 were added to the medium compared to maltodextrin (Figure 3). The proliferation of *L. crispatus* increased significantly when XOS35 was added to the medium. The proliferation of the strains in the co-culture was higher when XOS35 or XOS95 was added compared to the non-supplemented media (Figure 3). Supplementation of maltodextrin to the medium did not cause any changes.

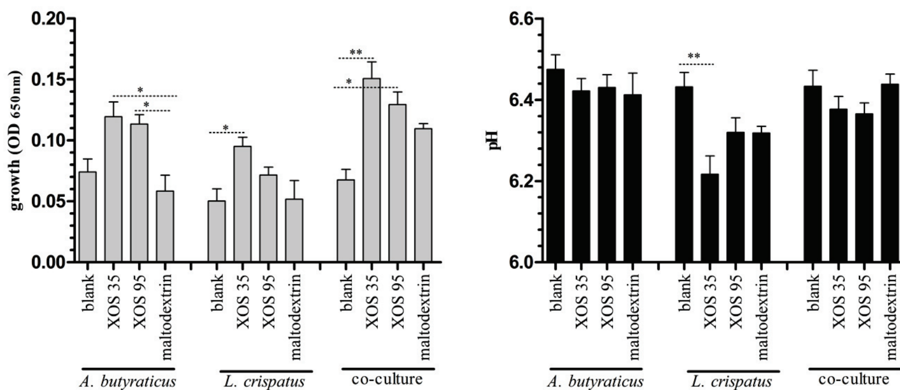

Figure 3. pH values and optical densities (650 nm) after 24h of *in vitro* fermentation of different substrates by *A. butyraticus, L. crispatus* and both in co-culture. All the *in vitro* fermentation experiments were done twice in triplicate. Statistical analysis was done with GraphPad Prism 5, using a Kruskal-Wallis test with a Dunns *post hoc* test. P-values ≤ 0.05 (*) and ≤ 0.01 (**) were considered significant.

The concentrations of the fermentation acids butyrate and DL-lactate were determined after incubation, in all monocultures and co-cultures (Figure 4). It was found that *A. butyraticus* was able to produce butyrate, while *L. crispatus* produced high concentrations of lactate. The concentration of butyrate or lactate produced by *L. crispatus* and *A. butyraticus,* respectively, were below the cut-off values (1mM and 0.5mM respectively) as determined during optimization of the HPLC method (De Baere *et al.*, 2013). XOS35 and XOS95 significantly stimulated lactate production by *L. crispatus* compared with *A. butyraticus,* which was not able to produce lactate. In the co-culture, lactate concentrations were very low, even when XOS35 or XOS95 were added to the medium, while the butyrate concentration was higher as compared to the concentrations in

the monoculture of *A. butyraticus* with XOS35 (3.3 ± 0.8 versus 2.3 ± 0.6, Figure 4), but non-significant. A similar observation was made for XOS95 (2.4 ± 0.8 versus 1.6 ± 0.6).

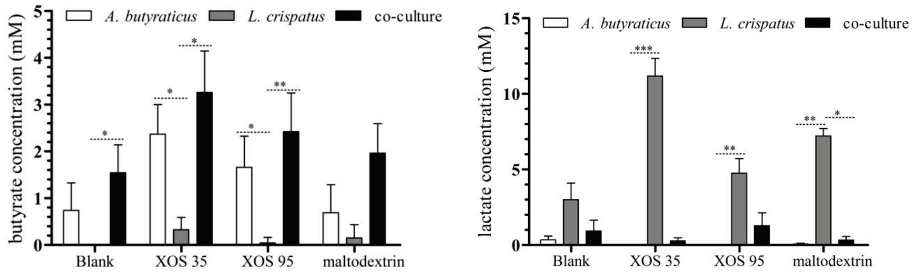

Figure 4. Butyrate and DL-lactate concentration after 24h of *in vitro* fermentation of different substrates by *A. butyraticus*, *L. crispatus* and both in co-culture. The *in vitro* fermentation experiments were done twice in triplicate. Statistical analysis was done with GraphPad Prism 5, using a Kruskal-Wallis test followed by a Dunns *post hoc* test. P-values ≤ 0.05 (*), ≤ 0.01 (**) and < 0.001 (***) were considered significant.

DISCUSSION

It is generally accepted that shifts in the intestinal microbiota composition may be the result of dietary changes, such as the addition of cereal fibres (Knarreborg *et al.* 2002; Shakouri *et al.* 2006; Torok *et al.* 2011). In the current study, we demonstrated that administration of XOS to broiler feed altered the microbiota composition in the gut, with butyrate-producing bacteria and lactobacilli being more abundant in caeca and colon, respectively. The family of the *Clostridium* cluster XIVa is significantly selected out of 35 families in the caecum and has a relative abundance of divergent family from 44.29% for the chickens feeding with XOS to 29.65% for the chickens without XOS. In the colon is the family of the *Lactobacillaceae* significantly selected out of 41 families and the relative abundance of divergent family is 81.54% for the chickens feeding with XOS and 41.73% for the one without XOS. At species level, 834 species were picked up in the caecum and 16 of them showed a significance difference with a relative abundance of 40.8% for the chickens fed with XOS and 32.14% for those without XOS. In the colon, 721 species were pick-up up and 11 of them showed a significance difference with a relative abundance of 79.04% for the chickens fed with XOS and 51.86% for those without XOS.

In the chicken gut, lactobacilli are one of the predominant genera (Wei *et al.*, 2013). These bacteria have the ability to adhere to the mucosal layers and epithelium, promoting colonisation (Kravtsov *et al.*, 2008; Sengupta *et al.*, 2013). Through interaction with the intestinal epithelial cells, lactobacilli can cause immunomodulation and offer protection to the intestinal barrier by antagonistic activities against pathogens (Rinttila & Apajalathi, 2013; Sengupta *et al.*, 2013; Servin, 2004). In addition, the probiotic use of lactobacilli has been shown to sometimes beneficially affect performance in broilers. Broilers fed diets containing a mixture of 12 *Lactobacillus* strains or a single *Lactobacillus acidophilus* strain had a better weight gain and a better FCR (Jin *et al.* 1998). Lactobacilli are known to ferment carbohydrates into lactic acid as major end-product which may lower the pH of the intestinal environment resulting in the inhibition of growth of acid-sensitive pathogenic bacteria. However, this pH effect may be rather limited as lactic acid is absorbed from the intestine or used as a substrate for lactate-utilizing bacteria, such as representatives of the genera *Eubacterium*, *Anaerostipes*, *Veillonella* and *Megasphaera* (Belenguer *et al.* 2007; Harmsen *et al.* 2002).

In the present study, in addition to the significant higher abundance of lactobacilli in the colon, we found an increased number of butyryl-CoA:acetate CoA-transferase gene copies in the caeca of chickens that received a XOS supplemented diet. Butyryl-CoA:acetate CoA-transferase is a key enzyme in the major pathway for bacterial butyrate production in the gut (Duncan *et al.* 2004). Hippe *et al.* showed that this enzyme is a suitable marker for the butyrate producing capacity of the intestinal microbiota which mainly belong to *Clostridium* cluster IV and XIVa (Hippe *et al.* 2011; Louis & Flint 2009). We observed a significant increase of members from both clusters in the caeca of chickens that were administered XOS.

The increased abundance of both lactobacilli and butyrate-producing bacteria can partly be explained by cross-feeding mechanisms. Bacteria related to *Eubacterium hallii* and *Anaerostipes caccae*, both members of *Clostridium* cluster XIVa, are able to convert acetate and lactate into butyrate (Duncan *et al.* 2004; Sato *et al.* 2008). This metabolic cross-feeding between lactate producing and lactate-utilizing bacteria may help to stabilize the luminal pH and may be a factor in the butyrogenic effect of certain dietary substrates (Belenguer *et al.* 2006). Our *in vivo* study showed a significant increase of the lactate-producing species *Lactobacillus crispatus* in the colon and the lactate-utilizing butyrate-producing species *Anaerostipes butyraticus* in the caeca. The sequence of the species of which the abundance was significantly different between the different groups showed 100% homology with the sequence of the type strains. The lactic acid produced by *L. crispatus* in the colon may reach the caecum and become available for *A. butyraticus* due to antiperistalsis (Hodgkiss, 1984; Janssen *et al.*, 2009). The *in vitro* fermentation assay showed that reference strains of both species metabolized XOS resulting in production of high concentrations of lactic acid by *L. crispatus*, which were supposed to be consumed by the butyrate-producing bacterium *A. butyraticus*. Most likely also many other strains can carry out a similar cross-feeding reaction in order to generate high butyrate levels in the chicken hindgut.

Production of butyrate most probably plays a role in the beneficial effects on gut morphology and growth performance observed in the current study. In poultry, butyrate enhances non-specific intestinal defence mechanisms against pathogens that can affect performance, such as *Clostridium perfringens*, by stimulating the mucin glycoprotein expression in intestinal epithelial cells (Gantois *et al.* 2006; Timbermont *et al.* 2010; Willemsen *et al.* 2003). Butyrate is a major energy source for the colonocytes and exerts anti-inflammatory activities by several mechanisms (Hamer *et al.*, 2008). One of these mechanisms is the suppression of nuclear factor kappa B (NF-κB) that regulates the expression of pro-inflammatory cytokines (Inan *et al.*

2000). Butyrate has also been shown to interfere with signalling by interferon-γ (IFN- γ) through its inhibitory effect on the activation of signal transducer and activator of transcription 1 (STAT1) (Klampfer *et al.* 2003). Butyrate also upregulates the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), a transcription factor that belongs to the nuclear hormone receptor family. PPAR-γ inhibits the expression of inflammatory cytokines and directs the differentiation of immune cells towards anti-inflammatory phenotypes (Martin, 2010; Schwab *et al.*, 2007; Wächtershäuser *et al.*, 2000).

We observed longer villi in the ileum of chickens that were fed a XOS-supplemented diet as compared to chickens fed a control diet. This effect on the small intestinal morphology may at least partly be due to butyrate production by *Clostridium* cluster IV and XIVa species in the hindgut through its effect on the expression of glucagon-like peptide-2 (GLP-2). Butyrate indeed appears to be a strong stimulator of GLP-2 production. This hormone is secreted by entero-endocrine L-cells and acts indirectly through multiple downstream mediators (Dube & Brubaker 2007). Its receptor (GLP-2R) is localized on distinct subpopulations of gut endocrine cells in the stomach, small intestine, and colon but also on subepithelial myofibroblasts (de Heuvel *et al.*, 2012; Drucker, 2001). Hu *et al*. showed a beneficial effect of intravenous GLP-2 injection in broilers on growth performance, intestinal morphology, villi height and crypt cell proliferation (Hu *et al.*, 2010).

In conclusion, XOS, supplemented to the broiler diet, improved broiler performance by improving the feed conversion ratio. Administration of XOS resulted in an increased abundance of butyrate-producing bacteria in the caeca and lactobacilli in the colon at day 26 of age. It is hypothesized that microbial cross-feeding, in which lactic acid produced by the lactobacilli is consumed by butyrate-producing bacteria in the caeca stimulates gut heath and consequently performance, through the beneficial effects of butyrate. Whether this cross-feeding also occurs in the complex gut ecosystem, needs to be clarified in further *in vivo* work.

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Chapter 3

Cellulose is not an inert feed supplement for broilers and improves performance during the starter period

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ABSTRACT

Dietary fibres are widely used to improve gut health and to stimulate performance in broiler chickens. Cellulose is a β-1,4-linked glucose polymer and a structural component of cell walls in green plants and vegetables. It is a non-fermentable fibre and considered inert. The aim of the current study was to analyze the effect of feed supplementation of cellulose on the performance and gut microbiota composition of broilers. Administration of cellulose to chickens, on top of a wheat-based diet, significantly improved feed conversion ratio (1.46 ± 0.02 vs. 1.37 ± 0.03, P = 0.04) and significantly increased daily weight gain (23.8 ± 0.48 g/day vs. 25.8 ± 0.36 g/day, P = 0.04) at day 13. No significant effects on performance were observed on day 26 and 39. The microbiota composition was determined using pyrosequencing of the 16S rRNA gene. At day 26, a significant higher relative abundance (%) of *Alistipes* genus was observed in the caeca of the broilers fed a cellulose-supplemented diet (5.86 ± 2.59 vs. 10.89 ± 3.42, P = 0.000165), compared to the animals fed a control diet. In conclusion, feed supplementation of cellulose influences broiler performance in the starter phase and it alters the microbiota composition within the phylum *Bacteroidetes,* specifically the *Alistipes* genus, at day 26. This suggests that cellulose is not essentially inert, and can alter the gut environment.

INTRODUCTION

The gastro-intestinal microbiota has an important role in chicken health and production. In a broiler chicken, the distal ileum, the caeca and the colon are regarded as fermentation chambers whose function is determined by the microbiota composition (Sekelja, et al., 2012). This chicken microbiota is dominated by the phyla *Firmicutes* and *Bacteroidetes* (Wei, *et al*., 2013). The majority of the sequences within the *Bacteroidetes* phylum belong to the genera *Bacteroides* and *Alistipes* (Kaakoesh, *et al*., 2014). The *Firmicutes* phylum is more diverse, and the majority of the sequences belong to different families, including *Ruminococcaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Peptostreptococcaceae* and *Streptococcaceae* (Kaakoesh, *et al*., 2014). This gut microbiota has been reported to be influenced by diet, age and environmental factors (Lu, *et al*., 2003; Torok, *et al.*, 2009; Torok, *et al*., 2008). In addition, there appears to be a clear link between the gut microbiota composition and the bird performance (Torok, *et al*., 2011a).

Dietary fibre consists of plant substances, including cellulose, hemicellulose, oligosaccharides, pectins and gums that resist hydrolysis by small bowel digestive enzymes (James, *et al*., 2003). As such dietary fibre represents a main energy source for the gastro-intestinal microbiota (Hamaker and Tuncil, 2014; Slavin, 2013). Studies have shown a correlation between consumption levels of dietary fibre and health benefits (Lattimer and Haub, 2010). The main areas of research on the effects of dietary fibre on poultry are related to its influence on rate of feed passage and organ size development; secretion of HCl, bile salts and digestive enzymes, and nutrient digestibility; feed intake and growth performance; and intestinal health and mucosa integrity, bird behavior, and microbial growth (Mateos *et al.*, 2012).

The dietary fibre cellulose is a linear chain of β-1,4-linked glucose monomers and a structural component of the cell wall in green plants and vegetables (Gilbert, 2010; O'Sullivan, 1997). *In vitro* incubation of human faeces with cellulose results in the formation of only small amounts of short-chain fatty acids (SCFA), due to the relative resistance of cellulose to bacterial degradation (Vince, *et al*., 1990). This was confirmed by another *in vitro* fermentation study (Johathan, *et al*., 2012), showing that the human gut microbiota is not adapted to ferment cellulose, although cellulose is present in various human food products. Consequently, cellulose can be used as a bulking agent in monogastric diets in order to decrease transit time or enhance water holding capacity (Hetland, *et al*., 2004; Montagne, 2003). A recent study confirmed that cellulose is essentially inert also in broilers as no nutritive value nor effects on growth were observed compared with the control animals fed silica sand as feed additive (Wils-Plotz, *et al*., 2013).

In broiler practice, however, beneficial effects of cellulose supplementation in broiler feed have been claimed. The study of Saki *et al.* showed that different ratio's of pectin and cellulose can differentially affect the intestinal condition and performance (Saki *et al.*, 2011). Until today, there is not much published research on the effect of cellulose on the gastro-intestinal health and performance of broilers. In the present study, we examined whether feed supplementation of cellulose could influence the broiler performance and the intestinal microbiota composition.

MATERIAL AND METHODS

ADDITIVE

The cellulose (Arbocel® B800, Rettenmaier & Söhne, Rosenberg, Germany) used in the *in vivo* study consisted of 99.5% amorphous cellulose and had an average fibre length of 130µm and an average fibre thickness of 20µm. The bulking density was within the range of 155 g/L and 185 g/L and the pH between 5.5 and 7.5.

ANIMALS AND DIETS

A total of 310 day-old Ross-308 male broiler chicks were housed on solid floor covered with wood shavings with 31 chicks per pen. Light schedule was set to provide 18h light and 6h dark. Animals were fed a wheat/mash-based diet of which the composition is shown in Table 1, no NSP enzyme (i.e. xylanase) were included. Sunflower meal and rapeseed meal were used as protein source in the diet; those proteins are slower digested than others. By using these protein sources, the diet is a suboptimal diet compared with the commercially available ones. In this diet also rye was used in order to increase the level of non-starch polysaccharides (NSP). All chickens received a starter feed from day 1 till day 13, a grower feed from day 14 till day 26 and a finisher feed from day 27 till day 39. For the chickens of the treatment group, the feed was supplemented with 0.5% cellulose during the starter period and 1.0% cellulose during the grower and finisher period. For this treatment group, four pens of 31 chickens were used. The animals from the control group were given the non-supplemented feed and this group consisted of six pens of 31 chickens. At day 13, 26 and 39, all broilers were individually weighed, as well as feed leftovers to calculate the feed conversion ratio (FCR). At day 26, 3 chickens of each pen were euthanized by intravenous injection of an overdose of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). The content of caecum and colon was collected and stored at -70°C until DNA was extracted.

Table 1. The composition and nutrient content of wheat-based diet administered to chickens. Start diet was given from day 1 until 13, grower was given from day 14 until 26 and finisher was given from day 27 until 39. **The treatment group received 0.5% cellulose on the top during starter and 1.0% cellulose during grower and finisher.**

	Starter diet	Grower diet	Finisher diet
Feedstuff (%)			
Wheat	50.48	55.00	58.94
Soybean meal (48)	21.12	15.22	11.51
Soybeans	5.00	5.00	5.00
Sunflower meal 27	5.35	6.00	6.00
Rapeseed meal	7.50	7.50	7.50
Animal fat	5.63	6.68	6.70
Soy oil	2.80	2.82	2.61
Vitamin + trace elements	1.00	1.00	1.00
$CaCO_3$	0.32	0.30	0.34
Di-Ca-phosphate	1.55	1.25	0.95
NaCl	0.21	0.25	0.25
Na-bicarbonate	0.16	0.10	0.06
L-Lys-HCl	0.31	0.35	0.39
DL-Methionine	0.26	0.24	0.23
L-Threonine	0.09	0.10	0.11
Phytase	0.02	0.02	0.02
Calculated nutrient composition (% as fed)			
Crude protein	21.50	19.60	18.27
Crude fat	9.00	10.00	10.00
Crude fibre	4.80	4.67	4.53
Non-starch polysaccharides	18.07	17.64	17.35
Metabolisable energy (MJ/kg)	11.65	12.10	12.30
Starch	29.0	31.5	33.7
Lysine, digestible	1.15	1.05	1.00
Sulfur amino acids, digestible	0.86	0.79	0.75
Threonine, digestible	0.75	0.68	0.65
Valine, digestible	0.80	0.76	0.70
Arginine, digestible	1.24	1.10	1.00
Isoleucine, digestible	0.74	0.66	0.60
Leucine, digestible	1.35	1.22	1.10
Ca	0.91	0.82	0.75
Available P	0.40	0.35	0.30
NaCl + KCl (mEq/kg)	240	207	182
Linoleic acid (18:2)	2.17	2.28	2.23

The difference between the calculated and measured (Weende analysis) value for protein was + 7.3% in starter, + 1.0% in grower and + 3.9% in finisher diet. For fat it was + 0.1% in starter, - 4.1% in grower and + 6.8% in finisher diet.

MICROBIOTA COMPOSITION

DNA Extraction

DNA was extracted from caecum and colon content from 3 chickens of each pen, using the CTAB method (Griffiths *et al.*, 2000; Kowalchuk *et al.*, 2000). To 100mg intestinal content, 0.5 g unwashed glass beads (Sigma-Aldrich, St. Louis, United States), 0.5ml CTAB buffer (hexadecyltrimethylammonium bromide 5% (w/v), 0.35 M NaCl, 120 mM K_2HPO_4) and 0.5 ml phenol-chloroform-isoamyl alcohol mixture (25:24:1) (Sigma-Aldrich) were added followed by homogenization in a 2ml destruction tube. The samples were shaken six times for 30 seconds using a beadbeater (MagnaLyser, Roche, Basel, Switzerland) at 6000rpm with thirty seconds between shakings. After 10 min at 8000rpm centrifugation, 300µl of the supernatant was transferred to a new tube. The rest of the tube content was re-extracted with 250µl CTAB buffer and again homogenized with a beadbeater. The samples were centrifuged for 10 minutes at 8000rpm and 300µl supernatant was added to the first 300µl supernatant. The phenol was removed by adding an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma-Aldrich) and a short spin. The aqueous phase was transferred to a new tube. The nucleic acids were precipitated with two volumes of PEG-6000 solution (polyethyleenglycol 30% (w/v), 1.6M NaCl) for two hours at room temperature. After 20 min at 13000rpm centrifugation, the pellet was rinsed with 1ml of ice-cold 70% (v/v) ethanol. The pellet was dried and resuspended in 100µl RNA free water (VWR, Leuven, Belgium).

16S rRNA gene sequencing

For each caecum and colon sample (9 chickens of each group), 16S rDNA PCR libraries specific for bacteria were generated with the primers E9-29 and E514-430 (Brosius *et al.*, 1981) targeting hyper variable regions V1-V3. The 454 Life Sciences's sequencing oligonucleotide design included two different titanium adapters named A or B (Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs) fused to the 5' end of each primer. The amplification mix contained 5U of FastStart high fidelity polymerase (Roche Diagnostics), 1x enzyme reaction buffer, 200µM dNTPs (Eurogentec, Liège, Belgium), 0.2µM of each primer and 100ng of genomic DNA in a volume of 100 µl. Thermocycling conditions consisted of a denaturation at 94 °C for 15 min followed by 25 cycles each of 94°C for 40 sec, 56°C for 40 sec, 72°C for 1 min and a final elongation step of 7 min at 72°C. These amplifications were performed on an Ep Master system gradient apparatus (Eppendorf, Hamburg, Germany). The PCR products were run on a 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using the SV PCR purification kit (Promega Benelux, Leiden, The Netherlands). The quality and

quantity of the products were assessed with a Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium). All libraries were run in the same titanium pyrosequencing reaction using Roche MIDs. All amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche, Vilvoorde, Belgium), the sequence number of each sample was normalized to 1836 reads.

The 16S rDNA sequence reads were processed with the MOTHUR package (Schloss *et al.*, 2009). The quality of all sequence reads were denoised using the Pyronoise algorithm implemented in MOTHUR and filtered with the following criteria: minimal length of 425bp, an exact match to the barcode and one mismatch allowed to the proximal primer. The sequences were checked for the presence of chimeric amplifications using Uchime (Edgar *et al.*, 2011). The resulting read sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database 1.15 of full-length rDNA sequences (http://www.arb-silva.de/) implemented in MOTHUR (Pruesse *et al.*, 2007). The final reads were clustered into OTUs using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cut-off. At the OUT level of analysis (OUT definition level for a 0.02 distance matrix), a total of 5967 OTUs were created. A taxonomic identity was attributed to each OTU by comparison with the SILVA database (80% homogeneity cut-off).

As a secondary analysis all unique sequences for each OTU were compared to the SILVA dataset 1.15 using BLASTN algorithm (Altschul *et al.*, 1990). For each OTU, a consensus detailed taxonomic identification was given based upon the identity (less than 1% of mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not). The raw sequences were deposited in Genbank (accession number pending).

Quantitative PCR

The number of *Alistipes* bacteria in the caeca of 3 chickens per pen was determined via qPCR using forward (5'-TTAGAGATGGGCATGCGTTGT-3') and reverse (5'-TGAATCCTCCGTATT-3') primers (Vigsnaes *et al.*, 2012). Amplification and detection was performed using the CFX384 Biorad detection system (Biorad, Nazareth-Eke, Belgium). Each reaction was done in triplicate in a 12µl total reaction mixture using 2x SensiMix™ SYBR No-ROX mix (Bioline, Kampenhout, Belgium), 3.0µM final primer concentration and 2µl of (50 ng/µl) DNA. The amplification program consisted of 1 cycle at 95°C for 10 min followed by 40 cycles of 15 sec at 95°C, 30 sec at 52°C and 20 sec at 72°C. The fluorescent signal was detected at the last step of each cycle.

STATISTICAL ANALYSIS

GraphPad Prism software version 5 was used to perform the statistical analysis of the performance data except for body weight. Differences in feed conversion ratio, feed intake and daily weight gain between the treatment and the control group were analyzed with an independent samples t-test. The body weight and qPCR were analyzed by means of a linear mixed effect model with pen included as random effect, using S-Plus. The differences were considered statistically significant at P value ≤ 0.05 and considered as a tendency at P value ≤ 0.1.

Results

Performance

The broiler performance data are shown in Table 2. During the starter period, supplementation of cellulose resulted in a significant increase of the daily weight gain (DWG) (P = 0.0473) and a significant decrease of the FCR (P = 0.0291). Feed supplementation of cellulose tended to increase body weight (BW) at day 13 (P = 0.0527). No significant differences were seen for DWG, FCR and BW at day 26 and 39 between the treatment and the control group. The feed intake did not differ significantly between the treatment and the control group during the whole trial. There are no differences in mortality between the two treatments.

Table 2. Effect of cellulose treatment on feed conversion ratio, body weight (g), daily weight gain (g/d/bird) and feed intake (g/d/bird) measured at three different time points. [1]values are presented as the mean ± standard error. Differences were determined by *t*-test. Means lacking a common superscript (within the same row) differ (P ≤ 0.05).

Intervals in days		FCR	BW (g)	FI (g/d)	WG (g/d)
0-13	**- cellulose**	1.46 ± 0.02	354 ± 4.34	34.7 ± 0.40	23.8 ± 0.48
	+ cellulose	1.37 ± 0.03	381 ± 4.80	35.4 ± 0.38	25.8 ± 0.36
	P-value	0.04 (*)	0.05	0.23	0.04 (*)
14-26	**- cellulose**	1.57 ± 0.01	1210 ± 14.27	103.9 ± 1.14	66.2 ± 0.97
	+ cellulose	1.55 ± 0.03	1258 ± 15.86	107.2 ± 1.18	69.3 ± 1.52
	P-value	0.48	0.18	0.07	0.11
27-39	**- cellulose**	1.82 ± 0.01	2545 ± 30.50	183.9 ± 1.39	101.1 ± 1.15
	+ cellulose	1.86 ± 0.02	2539 ± 35.64	183.5 ± 2.82	98.8 ± 2.06
	P-value	0.11	0.93	0.76	0.35
0-26	**- cellulose**	1.54 ± 0.01		69.3 ± 0.71	45.0 ± 0.68
	+ cellulose	1.50 ± 0.02		71.3 ± 0.44	47.6 ± 0.70
	P-value	0.11		0.07	0.05
0-39	**- cellulose**	1.66 ± 0.01		107.5 ± 0.85	64.8 ± 0.44
	+ cellulose	1.65 ± 0.02		108.7 ± 1.21	65.8 ± 0.98
	P-value	0.91		0.75	0.59

Microbiota Composition as Determined by 16S rRNA Sequencing and qPCR

At day 26, the intestinal microbiota in chickens is considered to be stable. Therefore caecum and colon content samples were taken at that time point to determine the composition. There were no significant differences in microbiota composition in the colon between the treatment and the control group. Supplementation of cellulose resulted in a significant increase of bacteria

belonging to the phylum *Bacteroidetes* in the caecum (P < 0.0001, Figure 1). This increase was the result of a specific significant increase in the family *Rikenellaceae* and within this family solely in the genus *Alistipes* (P < 0.05 and P < 0.0001, Figure 1). One unknown species (DQ456324) within this genus was significantly more abundant when cellulose was supplemented. A qPCR using primers that specifically amplify *Alistipes* bacteria confirmed the increase of the genus in the caecal microbiota of broilers fed the cellulose-supplemented feed (Figure 2).

The unknown species of the 16S sequencing data showed the highest 16S rRNA gene sequence similarity with *Alistipes putredinis* (96.48%) and *Alistipes finegoldii* (95.44%) using the EzTaxon database. *In vitro* culture of *Alistipes* spp., *Alistipes putredinis* and *Alistipes finegoldii* were shown to grow in the presence of cellulose as only carbon source (data not shown).

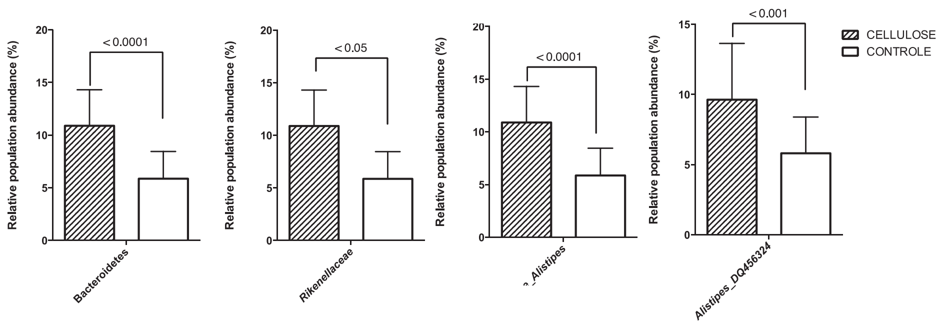

Figure 1. The bar charts showing relative population abundance (%) of the phylum *Bacteroidetes*, family *Rikenellaceae*, genus *Alistipes* and unknown species within genus *Alistipes* in the caecum at day 26.

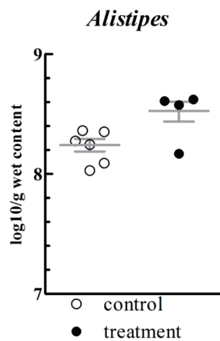

Figure 2. Number of *Alistipes* bacteria expressed as log10 copy number of the gene per g of wet caecal content of 26-day old chickens fed a wheat-based diet either or not supplemented with cellulose. The grey line shows the mean and standard error of the mean. ∘, control group; •, treatment group.

DISCUSSION

Dietary changes, such as the addition of dietary fibre, may result in a shift in the intestinal microbiota composition (Knarreborg, *et al*., 2002; Shakouri, et al., 2006). This has been shown for pectin, xylo-oligosaccharides and animal fat or soy oil (De Maesschalck, *et al*., 2015; Knarreborg, *et al*., 2002; Shakouri, *et al*., 2006). Cellulose is often regarded as an inert molecule that is considered to be poorly fermentable (Cummings and Englyst, 1987; Slavin, 2013). In the current study, we demonstrated that supplementation of cellulose to broiler feed can also induce microbiota composition shifts and improve performance, and is as such not inert.

In the cereal co-product hulls, cellulose is the predominant polysaccharide, followed by xylans and pectin substances (Knudsen, 2014). Natural cellulose can be divided into two groups: crystalline and amorphous components. They can be modified like powered cellulose, microcrystalline cellulose and hydroxypropylmethyl cellulose, which is then more easily broken down by the host into glucose and absorbed in the intestine (Lattimer and Haub, 2010; Takahashi *et al.*, 2003). In this trial modified cellulose was added to the broiler feed as amorphous powered cellulose. The cellulose concentration already present in the feed was calculated as less than 0.5% based on the acid detergent fibre (ADF) value, which is the sum of cellulose and lignin. During the trial 0.5% or 1% cellulose was added to the feed, suggesting that the effect in performance and microbiota composition may actually be due to the cellulose that was supplemented to the feed.

Cellulose is consisting of glucose units and is difficult to hydrolyse by cellulases (Mba Medie, et al., 2012; O'Sullivan, 1997). Cellulose, which suggest quite high concentration in grains typically fed to chickens, can only be utilized if this is first degraded into simpler forms by the microbiota residing in the gut (Stanley, *et al*., 2013). For example, two types of cellulases are able to liberate cellobiose, a disaccharide of glucose (Mba Medie, *et al*., 2012). After the degradation to smaller units, cellulose could eventually lead to glucose. Different studies showed that glucose plays an important role as energy source in young chickens (Moran, 2007; Noy and Uni, 2010). To break down cellulose, the gut microbiota thus needs specific enzymes that can degrade cellulose. In humans, members of the phylum *Bacteroidetes* harbour several genes encoding cellulases and xylanases suitable for the fermentation of cellulose and xylans (De Flippo, *et al*., 2010; Hamaker and Tuncil, 2014). In the present chicken trial administration of cellulose resulted in a higher abundance of the phylum *Bacteroidetes* and more specifically in a significant increase of the genus *Alistipes*. This genus harbours anaerobic, non-spore forming, non-motile Gram-negative

bacteria isolated from human faeces (Nagai, *et al.*, 2010; Rautio, *et al*., 2003; Song, *et al*., 2006). To identify patterns in the microbiota, Claesson *et al.* established co-abundance associations of genera and clustered correlated genera into six co-abundance groups (CAGs) (Claesson, *et al*., 2012). *Alistipes* spp. are considered to be part of the core microbiome of the human intestine as they belong to one of the CAGs (Claesson, *et al*., 2012). Wei *et al.* have shown that *Alistipes* species also represent more than 1% of the bacterial sequences in the chicken gut.

Alistipes strains have already been related to performance parameters of broilers. *A. finegoldii,* in the caeca, is shown to be associated with performance in broilers (Torok, *et al*., 2011b). The exact mechanism by which *A. finegoldii* could affect performance is still unclear. Cellulose degradation and fermentation to succinate may play a role. Indeed, in the present study, we showed that *Alistipes* spp. could grow on cellulose as carbon source. The major fermentation end-product of *Alistipes* bacteria is succinate (Rautio, *et al.*, 2003; Reichardt, *et al*., 2014). Succinate can provide energy in two distinct ways. First, it can be taken up directly by chicken intestinal cells through a sodium-dependent transport system (Kimmich, *et al*., 1991). Succinate can then directly be introduced in the tricarboxylic acid (TCA) or Krebs cycle. Secondly, it can be used by numerous other *Bacteroidetes*, and be converted to propionate after decarboxylation, which appears to be the most abundant route for propionate formation (Reichardt, *et al.*, 2014). Propionate in turn can be used as an energy source by the epithelial cells. Moreover, propionate has also health-promoting effects, such as an anti-inflammatory action that may influence performance (Hosseini, *et al*., 2011; Vinolo, *et al*., 2011).

During this study, a positive effect of cellulose was seen in young chicks by a decreased feed conversion ratio. During grower and finisher period, no effect was observed on performance of the chickens. The exact reason for that is not known. During starter period the chickens received 0.5% cellulose, which was increased to 1% in grower and finisher period. This was done because it was thought that the microbiota would adapt to cellulose during starter phase. The development of the microbiota composition is indeed a dynamic process and it can be manipulated by altering the diet. It is possible that the extra 0.5% during the grower and finisher phase does not fully compensate for the reduction of directly available energy from digestible ingredients as a consequence of the dilution effect. So further research is needed to determine why cellulose has no effect on the broiler performance from grower phase onwards. This can been done by setting up a new *in vivo* trial, in which the effect of different doses of cellulose is evaluated on performance and microbiota.

In conclusion, cellulose is able to improve performance of young broilers when supplemented to the broiler diet. Administration of cellulose resulted in an increased abundance of the *Alistipes* genus in the caeca. It is hypothesized that this genus stimulates gut health and consequently performance by producing succinate as end-product. Further research on cellulose degradation by the microbiota is needed to confirm this statement.

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General discussion

Butyrate appears to be an evolutionary highly conserved crucial interkingdom signalling molecule that is sensed by the host as a “green light” for activating intestinal digestion and absorption, as opposed to high concentrations of hydrogen sulphide and a number of other molecules, which serve as “red light” signals. Obviously, these molecules are terminal metabolites, which are common to certain specific categories of microbes in the intestinal tract. For a number of years, our research group has focused on the direct administration of these metabolites and the ensuing effects on susceptibility to certain pathogens, including *Salmonella*, *Campylobacter* and *Clostridium perfringens*. More recently, attention has shifted towards a better understanding of the microorganisms that are responsible for the massive endogenous production of butyrate in the lower gastro-intestinal tract (GIT) for the simple reason that the production capacity of the healthy microbiome largely exceeds what can be provided by direct feed supplementation of butyrate, even under the most optimal conditions of delivery.

This thesis fits well into the general strategy that aims at unravelling the mechanisms that protect the intestine from continuous life-threatening inflammation and allow for optimal digestion of nutrients. The part played by the microorganisms in these processes is crucial. In the first chapter of this thesis a modest contribution to knowledge of the intestinal microbiome is made using culturing techniques and characterization of some specific microbial species for several reasons explained below.

CHICKEN INTESTINAL MICROBIOTA INFLUENCES PERFORMANCE

In the last decade, the poultry industry has become the most dynamic sector within the global meat business as broilers can be produced in the shortest time (± 42 days) compared to other meat producing animals. Poultry meat is important to reduce the shortage of animal protein especially in developing countries (Neves *et al.*, 2014). When looking at the AVEC (Association of Poultry processors and poultry trade in the EU countries) annual report of 2014 (estimates April 2014) the world meat production for broilers and turkey is increased with 1.16% and the consumption with 1.46% (from 2013-2014). In 2013, Belgium produced 250 000 tons carcass weight of poultry meat and this is 2.4% of the broiler production in the European Union. Changes in management, such as optimization of temperature, lighting and bird densities, improvements in poultry nutrition and genetic selection for feed efficiency have resulted in the marketing of chickens in about half the time and at about twice the body weight as compared to 50 years ago (Barbut *et al.*, 2008). The study of Tuyttens described the evolution of performance between 1998 and 2008 (Tuyttens *et al.*, 2014). The slaughter age decreased from 41.3 to 40.1 days, whereas the average slaughter weight increased from 2.1 to 2.4kg. While the mortality decreased by 1.9% to an average of 3.9% (Tuyttens *et al.*, 2014). Little attention has been paid to the influence of all these changes on the gut microbiome although the composition of the microbiome composition has been shown to have a major influence on performance especially under conditions of intensive feeding. Indeed intensive (over)feeding can lead to disturbance of the intestinal microbiome, termed dysbiosis.

In the past, disturbance in the intestinal microbiome were kept under control by using growth-promoting antibiotics. They allowed improving feed conversion and animal growth and reduced morbidity and mortality due to clinical and subclinical diseases (Butaye *et al.*, 2003). However, concerns were raised that the use of these antibiotics could lead to increased resistance in bacteria, particularly in Gram-negative bacteria like *Salmonella* spp. and *Escherichia coli* (Butaye *et al.*, 2003). Since the European Union banned the use of growth promoting antibiotics in 2006, intense research efforts have been undertaken to find alternative tools to improve feed conversion and to protect intestinal health. One type of the alternatives are prebiotics, which facilitate the growth of an optimal microbiota (Choct, 2009; Nabizadeh, 2012; Sugiharto, 2014; Yang *et al.*, 2008).

A balanced intestinal microbiome is indeed important for bird performance. Torok *et al.* were the first to demonstrate a positive correlation between gut microbial communities and bird performance using terminal restriction fragment length polymorphism (T-RFLP) (Torok *et al.*, 2008). A limitation of this T-RFLP analysis has been the inability to reliably assign OTUs to phylogenetic groups. Nevertheless the presence of specific beneficial bacterial species and/or the absence of specific detrimental bacterial species were suggested to contribute to improved performance (Torok *et al.*, 2008). Recently, more comprehensive analyses using next generation sequencing (NGS) technology have revealed bacteria such as *Clostridium hylemonae*, *Lactobacillus celeohominis*, *Bacteroides fragilis* and *Ruminococcus* sp., to be associated with improved growth performance of broilers (Stanley *et al.*, 2014; Stanley *et al.*, 2013a; Stanley *et al.*, 2013b). The poultry industry may benefit from the identification of bacterial species, that promote bird performance (Geier *et al*., 2009) and use them as probiotics, which are considered nutritional tools that promote growth and meat quality by modulating the intestinal microbiota and inhibiting pathogens (Lutful Kabir, 2009).

The variability in growth performance of broilers has been a matter of interest and investigation for farmers. Chickens have been selected based on different parameters including high feed intake. The body weight gain of broilers is closely linked to host genetics, diet, age and rearing environment (Lu *et al.*, 2003; Singh *et al.*, 2014; Torok *et al.*, 2009). Host genetics associated with weight gain and feed conversion efficiency have been shown to play a role in changes in the gut physiology and microbial structure of chickens (Lumpkins *et al.*, 2010; Singh *et al.*, 2014). It is currently unclear whether any parallel can be drawn between the microbiota associated with obesity in humans and the microbiota associated with improved energy harvesting and increased FCR in production animals such as broiler chickens (Rinttila & Apajalathi, 2013). In humans, it is well established that the ratio between *Firmicutes* and *Bacteroidetes* correlates with body weight as the ratio is shown to be significantly higher in obese individuals (Ley *et al.*, 2006). The potential performance-stimulating bacteria identified in another study by Torok *et al.* belong predominantly to the phylum *Firmicutes*, and to a lesser extent to the phyla *Bacteroidetes* and *Proteobacteria* (Torok *et al.*, 2011). This study, however, did not look at the ratio between *Firmicutes* and *Bacteroidetes*, as bacteria were not quantified. Instead, they looked in more detail to bacteria that could affect the performance and identified 10 species including, *Lactobacillus salivarius*, *L. crispatus*, *L. aviaries*, *Escherichia coli* and *Gallibacterium anatis* in the ileum and *Faecalibacterium prausnitzii*, *Clostridium lactatifermentans*, *Ruminococcus torques* and *Alistipes finegoldii* in the caeca (Torok *et al.*, 2011). The three *Lactobacillus* spp. in the ileum were

associated with poor performance. In our XOS trial, we showed an improved performance with XOS and a significant increase of lactobacilli in the colon. In the XOS trial, the generated lactate was probably very efficiently converted into butyrate by the expansion of the *Anaerostipes butyraticus* population. Further in the study of Torok *et al. A. finegoldii* was associated with either improved or poorer performance. In the present thesis, adding cellulose had a beneficial effect on daily gain in association with a significant increase of the *Alistipes* genus. Therefore manipulation of the microbiota to produce a community most conducive to optimal performance will be one of the key considerations when designing prebiotic-containing feeding regimes. However, the question of how the intestinal bacterial community relates to relevant metabolic changes and to broiler performance is not completely understood. The best strategy would be that all future studies looking at performance would also study the intestinal microbiota and their metabolites.

The intestinal microbiota creates a complex environment within the intestine that affects many host functions such as digestion and fermentation of dietary compounds with a direct consequence for gastro-intestinal development; the regulation of intestinal epithelial proliferation, inflammatory immune responses and host energy metabolism; synthesis of vitamins; and filling of a microbiological niche that might otherwise be colonized by potentially harmful enteric microorganisms (Flint *et al.*, 2012; Guarner, 2006; Klasing, 2007; Rakoff-Nahoum *et al.*, 2004; Rinttila & Apajalathi, 2013; Walker *et al.*, 2011). A favorable microbiota should have a balance between genera, which are not responsible for inflammation and have the possibility to produce SCFA by carbohydrate fermentation. Different studies, including our, have shown that in feed supplementation of prebiotics promote growth of specific members of the resident gut microbiota (Scott *et al.*, 2013). The characterization of the intestinal microbiota is critical to understand the influence on growth performance, health and energy uptake.

THE IMPORTANCE OF CHARACTERIZING UNKNOWN SPECIES

Around 90% of the bacteria in the GIT are unknown species, indicating that the knowledge of the intestinal microbiota is incomplete (Apajalahti *et al.*, 2004; Bjerrum *et al.*, 2006; Gerritsen *et al.*, 2011). Microorganisms recovered by cultivation techniques represent only a small fraction of the total diversity that exists in the gut because of difficulties encountered during isolation and cultivation such as oxygen sensitivity, temperature, pH, the need for specific substrates etc. (Gaskins *et al.*, 2002; Oakley *et al.*, 2014; Rappe & Giovannoni, 2003). During the last decade, several technical advances such as direct sequencing and metagenomic approaches have allowed insights into the uncultured majority (Oakley *et al.*, 2014; Qin *et al.*, 2010). Most studies on microbial communities in systems ranging from the open ocean and soil to the gut have depended on a single gene, the 16 small subunit ribosomal RNA (16S rRNA) gene (Costello *et al.*, 2009; Gilbert *et al.*, 2012; Nemergut *et al.*, 2011; Poretsky *et al.*, 2014). These 16S rRNA fragments allow the investigation of bacterial community structures, phylogenetic composition and species diversity. In addition these data may provide some information on the metabolic capacity and thus functional diversity based on the known characteristics of their closest phylogenetic neighbors (Shah *et al.*, 2011; Streit & Schmitz, 2004). The use of either phylogenetic or functional genes has improved our knowledge about the ecology of microbial functional groups (Salles *et al.*, 2012).

Although new developed technologies such as metagenomics and metatranscriptomics provide more information on functionality of the microbial community, it is still important to isolate and cultivate single microbial species in order to gain knowledge and better understanding of microbial physiology and to use isolates for various biotechnological applications (Gao *et al.*, 2013). Since metatranscriptomics shed light on microbial metabolism *in situ* it may provide critical clues for directing the culturing of hitherto uncultured microorganisms (Bomar *et al.*, 2011). Nevertheless, the obtained 16S rRNA sequences are compared to reference data sets of the corresponding region derived from the SILVA database and are clustered into OTUs using the nearest (i.e., single-linkage) neighbour hierarchical clustering algorithm with a specific distance unit cut-off, this means that all sequences within a specified threshold of any other sequence belong to the same OTU (Schloss & Westcott, 2011). When those OTUs represent unknown species they are seldom used for further investigation. One of the reasons may be that the unknown species occur only at limited concentration and therefore might be less relevant, but another probably more important reason is that no characteristics of their functional role in the environment are known. The classification and characterisation of newly isolated bacteria not

only results in a name but also more importantly provides information on the metabolic capacities that help to explain their role or to set up some hypotheses for further research based on their function.

ERYSIPELOTRICHACEAE, A FAMILY WITH SEVERAL NEW GENERA

The majority of sequences within the *Firmicutes* phylum belong to the families *Ruminococcaceae* and *Lachnospiraceae*, the so-called *Clostridium* cluster IV and XIVa, respectively (Collins *et al.*, 1994). The bulk of butyrate-producing bacteria belong to one of these two families. Previously only a few butyrate-producing strains from chicken origin were cultured (Bjerrum *et al.*, 2006). Recently Eeckhaut *et al*. sampled chicken caecal content and isolated 16 butyrate-producing strains with a unique 16S rRNA gene sequence that were dispersed among *Clostridium* clusters IV, XIVa, XIVb and XVI (Eeckhaut *et al.*, 2010). In this study butyrate-producers related to cluster XVI were suggested to play a more important role in the chicken than in the human gut.

Members of *Clostridium* cluster XVI or family *Erysipelotrichaceae* are bacteria with incoherent cell morphology that produce acid but no gas from glucose or other carbohydrates and normally have a respiratory or fermentative metabolism (Collins *et al.*, 1994; Verbarg *et al.*, 2014; Verbarg *et al.*, 2004). At the beginning of 2014, the family *Erysipelotrichaceae* consisted of 13 genera and most of them harboured one species: *Allobaculum*, *Bulleidia*, *Candidatus Stoquefichus*, *Catenibacterium*, *Coprobacillus*, *Dielma*, *Eggerthia*, *Erysipelatoclostridium*, *Erysipelothrix*, *Holdemania*, *Kandleria*, *Solobacterium* and *Turicibacter.* They all are non-motile and non-spore forming Gram-positive bacteria except for the *Dielma* genus with species *Dielma fastidiosa* which is a motile Gram-negative bacterium (Bosshard *et al.*, 2002; Downes *et al.*, 2000; Greetham *et al.*, 2004; Kageyama & Benno, 2000a, b; Pedersen *et al.*, 2011; Pfleiderer *et al.*, 2013; Ramasamy *et al.*, 2013; Salvetti *et al.*, 2011; Verbarg *et al.*, 2014; Verbarg *et al.*, 2004; Willems *et al.*, 1997; Yutin & Galperin, 2013). All genera within this family grow facultative or obligate anaerobically except for bacteria of the genus *Erysipelothrix,* which are also able to grow aerobically (Verbarg *et al.*, 2004). Most species are phenotypically similar, so they are differentiated based on their 16S rRNA gene sequence.

Streptococcus pleomorphus, Eubacterium cylindroides and *Eubacterium biforme* are known as members of the *Erysipelotrichaceae* family, however, it is obvious that they are misnamed because at the time they were classified the methods for classification were not as extensive as they are now (Collins *et al.*, 1994). In our study, they were renamed as *Faecalicoccus pleomorphus*, *Faecalitalea cylindroides* and *Holdemanella biformis*, respectively. During this study, also a new isolate was characterized and described as *Faecalicoccus acidiformans*.

Recently, another new genus *Catenisphaera* has been described as the first genus within the family *Erysipelotrichaceae* isolated from a methanogenic reactor fed with food waste instead of intestinal content (Kanno *et al.*, 2015). This brings the total to 17 genera within the family *Erysipelotrichaceae*. Classification ensures that certain properties can be linked to particular species or even genera. For example, it has been shown that all genera have the ability to produce lactate, but only 6 genera *Allobaculum*, *Catenibacterium*, *Catenisphaera*, *Faecalicoccus*, *Faecalitalea* and *Holdemanella* have the ability to produce butyrate (De Maesschalck *et al.*, 2014; Greetham *et al.*, 2004; Kageyama & Benno, 2000b). This difference in properties can be important for further research.

Within this family, each species appears to have its own metabolic characteristics. Despite the fact that these characteristics are described, the exact function and the abundance of the species in the gut is not yet known. So further research is necessary to determine and to understand their functions in the gut. Few data are available so far. Indeed, a study that compared different kits to extract DNA from human stools showed that the family *Erysipelotrichaceae* is part of the human gut microbiota (Kennedy *et al.*, 2014). The *Erysipelotrichaceae* family was shown to be increased in pigs when feeding resistant starch (RS) (Haenen *et al.*, 2013). This study focused on changes of the microbiota composition and SCFA concentration and showed that bacterial groups belonging to *Clostridium* cluster IV, IX, XV and XVII as well as propionate-producing microorganisms increased in RS-fed pigs, but that RS fed pigs also had a significantly increased concentration of total SCFA, acetate, propionate and valerate in the caecum and the colon. In our study we showed a significant reduction in the presence of *Erysipelotrichaceae* in the caeca of broilers fed XOS (4.5% versus 2.6%, data not shown) although an *in vitro* fermentation experiment showed that *Faecalitalea cylindroides*, *Faecalicoccus pleomorphus*, *Holdemanella biformis* and *Allobaculum stercoricanis* were able to grow and produce SCFA in the presence of XOS35 (data not shown). This may suggest that *Clostridium* cluster XVI bacteria do belong to the gut microbiome of the chicken but that they may be outcompeted by other genera (*Lactobacillus* and *Anaerostipes*) in the presence of certain substrates. This probably means that the redundancy in microbial populations producing butyrate allows the intestinal microbiota to dynamically and rapidly adapt to changes in diet.

DIET AND PREBIOTICS

Diet is indeed a main contributor to broiler performance and affects the microbial community (Gong *et al.*, 2002; Knarreborg *et al.*, 2002; Lu *et al.*, 2003; Pan & Yu, 2014; Zhu *et al.*, 2002). Wheat is a very variable grain in terms of its physical and chemical characteristics, and has great influence on broiler performance and nutrient digestibility (Amerah, 2015). Also the source and level of protein has a major effect on growth performance. Moreover, it is the most expensive component in broiler diets (Dirain & Waldroup, 2002; Zaham *et al.*, 2008). In one of our *in vivo* trials, we compared a wheat-based diet supplemented with either slow or fast fermentable protein. Sunflower meal and rapeseed meal were added as slow fermentable protein. These proteins are expected to be partly available in the lower digestive tract. In contrast, potato proteins and corn gluten were added as fast fermentable proteins, to be absorbed in the upper digestive tract. Slow fermentable proteins were shown to have a negative influence on performance in terms of FCR, growth and body weight. Although there were significant differences in performance, no significant differences in abundance of *Firmicutes*, *Bacteroidetes*, *Clostridium* cluster I, IV, XIVa, *Enterobacteriaceae* and *Lactobacillaceae* could be found when quantified by qPCR. Besides the microbiota composition, also other parameters may influence performance. Effects on intestinal morphology could be analysed by measuring the villi length and thickness of the tunica muscularis. The presence of inflammation could be analysed by measuring the abundance of T-lymphocytes, heterophils, macrophages, etc. These parameters were not investigated in this trial, so the reason for the change in performance between slow and fast fermentable proteins is still unclear. Further research is necessary to explain these performance data.

PERSPECTIVES OF XYLO-OLIGOSACCHARIDES AS PREBIOTICS TO INFLUENCE BROILER INTESTINAL HEALTH

Prebiotics are defined as ingredients that improve host health (Gibson & Roberfroid, 1995; Gibson *et al.*, 2004) and are therefore used as feed additives in broiler feed in an attempt to improve both health and performance of chickens (Hajati & Rezaei, 2010). The farmers and poultry industry are interested in healthy chickens, but above all in an increased meat yield with a lower cost. In order to keep the cost low, there must be taken into account, the dose and the duration of the addition of the prebiotics or dietary fibres. If more than 2% is added to the feed, the total nutrient content will be exceeded. In addition, these dietary fibres and prebiotics have no direct effect on the host, because this involves a shift in the microbiota composition and so the effects will be seen later on. The dose does have an impact on the performance result, because we have also looked at 1% XOS and there we saw that the broilers had an increased FCR compared

to the control (data not shown). The reason for that was not further explored. If the duration needs to be adjusted because of the high cost, then the prebiotic or dietary fibre should be added to the diet during the first half of the starter and the grower period. During both periods, the microbiota is most formed or edited on the basis of the diet. As such several dietary fibres are tested for their influence on intestinal health by looking at the microbial composition and the metabolites they produce.

To our knowledge XOS is still classified as a prebiotic candidate (Mäkeläinen *et al.*, 2009). We showed an improved performance at slaughter age for broilers that received XOS. Improved performance with XOS has been confirmed in another study using broilers (Zhenping *et al.*, 2013). For the classification of XOS as prebiotic, it is necessary to review the available data and evaluate whether the three criteria are fulfilled (Gibson *et al.*, 2004). These criteria are (1) resistance to gastric acidity, to hydrolysis by enzymes and to gastro-intestinal absorption; (2) fermented by intestinal microbiota and (3) selectively stimulating the growth and/or activity of intestinal bacteria that contribute to health and well-being.

A study of Courtin *et al.* showed resistance of XOS towards gastric acidity (Courtin *et al.*, 2009). They mimicked gastric conditions (pH 2, 37°C) *in vitro* and found less than 10% hydrolysis of XOS even after 14 days (Courtin *et al.*, 2009). Endoxylanases and xylosidases have been shown to hydrolyse XOS, however no reports of mammalian endoxylanases and xylosidases have been published, which suggests that XOS are non-digestible (Broekaert *et al.*, 2011).

Different *in vitro* and *in vivo* studies showed the possibility of XOS fermentation by intestinal microbiota (Broekaert *et al.*, 2011). In our studies we showed the fermentation of two concentrations of XOS (35% and 95%) by chicken caecal and colonic inocula with the production of SCFA (data not shown). *In vitro* fermentation of XOS showed the production of SCFA, which has been corroborated by several *in vivo* studies (Broekaert *et al.*, 2011). The differences in the SCFA produced can be dependent on the diet. A particular diet has direct and indirect impact on the intestinal microbiota. When the host receives XOS as a feed supplement, this may have different effects depending on the intestinal microbiota. In our *in vivo* study XOS was supplemented to a wheat-based diet with slow fermentable proteins, which is a sub-optimal diet. Supplementation of XOS resulted in an increase of butyrate-producing bacteria in the caeca and an increase of lactate-producing bacteria in the colon. Taking into consideration the physiological anti-peristalsis in the chicken intestine, the function of the chicken colon may be considered to some extent overlapping with the chicken ileum. Both *in vitro* and *in vivo* tests

demonstrate that the intestinal microbiota has the ability to consume and ferment XOS, which could influence the well-being of the host.

As a consequence of its fermentation by intestinal microbiota, the XOS prebiotic selectively stimulates the growth and the activity of important intestinal bacteria such as lactate- and butyrate-producing species. Several studies have demonstrated that different bifidobacteria and *Lactobacillus* species, can efficiently utilize XOS (Broekaert *et al.*, 2011). A number of *in vivo* intervention trials in animals and humans have established that XOS has strong bifidogenic properties (Broekaert *et al.*, 2011). Several studies using rats, mice and chickens showed increases in colonic and caecal *Bifidobacterium* spp. following XOS administration (Courtin *et al.*, 2008; Hsu *et al.*, 2004; Santos *et al.*, 2006). Our *in vivo* trial showed an increased level of lactobacilli instead of bifidobacteria, which may have different explanations. First of all, the feed composition that we used in our trial may be more favourable for lactobacilli. Secondly, the intestinal microbiota of chickens develop not always in the same manner as that of mammals because different environmental factors have an influence on the intestinal microbiota development. In addition the study of Scott *et al.* showed that XOS is also a growth substrate for butyrate-producing species of *Clostridium* cluster IV and XIVa (Scott *et al.*, 2014). Our *in vitro* fermentation assay with XOS and *L. crispatus* showed that this species has the potential to grow in the presence of XOS. The lactate produced by *L. crispatus,* could be used for cross-feeding by lactate-utilizing bacteria like *Anaerostipes butyraticus*, which has the ability to produce butyrate from lactate (Duncan *et al.*, 2004).

The results of the *in vitro* and *in vivo* experiments using XOS show us that XOS indeed may fulfil all three criteria for the prebiotic classification, thus XOS should be classified as a prebiotic.

COULD CELLULOSE BE MORE THAN JUST AN INERT INSOLUBLE FIBRE?

The profit of the poultry industry depends mainly on the nutritive value and the cost of the feed (feed cost is about 70% of the total cost of intensive poultry production system); therefore one searches for new dietary fibres that can reduce the cost without impact on the nutritive value. Although cellulose was previously used as a bulking agent in monogastric diets (Hetland *et al.*, 2004; Montagne, 2003), we showed that cellulose could influence broiler performance during the starter phase up to 13 days of age. This suggests that cellulose could be an important dietary fibre to get a good start for broilers. How come the effect of cellulose has not been noted before? Effects may have been masked by different factors including diet composition, pelleted vs. mash feed, concentration of substrate, source of substrate, etc. In our trial, a wheat/mash-based diet

with slow digestible protein like sunflower and rapeseed meal was used. Mash feed has the advantage that heat sensitive endogenous enzymes that break down the non-starch polysaccharides (NSP) are still present (Amerah, 2015; Scott *et al.*, 2003). Pellets on the other hand have the advantage that they can be taken up very fast which results in an increased feed uptake (Abdollahi *et al.*, 2013).

The diet used in this *in vivo* trial had a high protein level by using wheat supplemented with slow fermentable proteins. To investigate the possibility of reducing dietary proteins and energy, Tahir *et al.* compared different feed enzymes such as cellulase, hemicellulase and pectinase but also multi-enzyme preparations (Tahir *et al.*, 2008). In this study, the feed enzymes were added to the diet and they showed that the mixed enzyme preparation could effectively degrade indigestible cell constituents and thus enable the protein of the broiler feed to become more digestible. Therefore it could be of interest to search for bacterial species that have these enzymes present in their genome and could help to consume dietary proteins. Our cellulose trial showed an increased abundance of the *Alistipes* genus and the Carbohydrate-Active-Enzyme database (CAZy) showed the presence of at least one cellulase enzyme in the species *A. finegoldii* and *A. shahii*. This indicates that adding cellulose to the diet is responsible for the increase the presence of the genus *Alistipes* and influences the digestion of dietary protein present in the diet. This digestion of proteins can improve the broiler performance of this sub-optimal diet. In our *in vivo* trial, we supplemented cellulose to broiler feed and it induced microbiota composition shifts and improved performance, suggesting that cellulose is not essentially inert.

FUTURE PERSPECTIVES

Recently, more bacteria of the family *Erysipelotrichaceae* or *Clostridium* cluster XVI were isolated and characterized, which shows an increased interest in this family. Some studies have demonstrated that they belong to the normal intestinal microbiota of the host and most of them produce lactate as major fermentation product and some of them are also able to produce butyrate. Until today, there is very little known about the effect of this family on gut health. Are they beneficial because of their lactate and/or butyrate production or by other mechanisms?

Based on the results of the XOS trial we hypothesized a microbial cross-feeding in which lactic acid produced by the lactobacilli is consumed by butyrate-producing bacteria, which then stimulate gut health and consequently performance. This microbial cross-feeding between *Lactobacillus crispatus* and *Anaerostipes butyraticus* needs to be clarified in new *in vivo* experiments. For example, this can be done by setting up a new performance experiment where we compare the XOS group with three other treatment groups. The first group would receive *A. butyraticus*; the second one *L. crispatus* and the third group both species. At different time points, the performance parameters can be measured as also the number of bacteria and the SCFA concentration in the caecal and colonic content. Today XOS are described as a prebiotic candidate, although different *in vivo* and *in vitro* studies show that XOS fulfil all three criteria for the prebiotic classification. This suggests that XOS needs to be classified as prebiotic instead of prebiotic candidate.

Further investigation is necessary to classify cellulose as dietary fibre instead of an inert component. First different *in vitro* fermentation experiments need to be set-up, in order to get an overview of the intestinal bacteria that are able to ferment cellulose, focusing on the end-products of their metabolism, including SCFA, succinate, lactate, gases etc. As the *Alistipes* genus is only recently established, it would be interesting to know more about this genus. The description showed that this genus is able to produce succinate and the study of Reichardt *et al.* suggests that succinate could be converted to propionate after decarboxylation in the succinate pathway (Reichardt *et al.*, 2014). This pathway is present in the abundant phylum *Bacteroidetes* as well as in several *Negativicutes* bacteria. It would be interesting to prove this possible cross-feeding by *in vitro* fermentation assays, and study more in depth the effect of succinate and propionate on gut health parameters.

CONCLUSION

The family *Erysipelotrichaceae* or *Clostridium* cluster XVI is a relative new identified family in the host intestine and is relatively abundant. Therefore further research is necessary to understand their function in intestinal health. In addition to the description and isolation of new bacterial species in the intestine, it is even more important to stimulate beneficial bacteria in order to improve the health of broilers. Prebiotics or dietary fibres could do this. An *in vivo* trial with XOS has demonstrated an improved performance at slaughter age which may be explained by an increased number of butyrate-producing bacteria in the caecum and an increased number of lactate-producing bacteria in the colon after 16S rRNA sequencing and qPCR analyses. This suggests that XOS could be an interesting prebiotic to improve broiler performance by stimulating butyrate-producing bacteria. A second *in vivo* trial with cellulose has demonstrated an improved performance in the early life and an increased number of *Alistipes* species in the caecum after 16S rRNA sequencing and qPCR analyses. This suggests that cellulose is more than an inert component. Further research is necessary to classify cellulose as a dietary fibre.

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Appendix

SUMMARY

Increasing public concern about the use of antibiotics in the poultry industry has influenced the ways in which poultry producers are working towards improving birds' intestinal health. With the scientific information currently available, there is no doubt that the intestinal microbiota is directly or indirectly involved in all physiological and pathological processes that occur in the digestive tract and even in many processes outside the digestive tract and thus play a vital role in host welfare and productivity. The gastro-intestinal tract has the largest microbial population and is dominated by two bacterial phyla, *Firmicutes* and *Bacteroidetes*. Products of microbial metabolism act as signalling molecules and influence the host's metabolism. More specifically protein fermentation results in the production of more detrimental metabolites for the host, while fermentation of carbohydrates results mainly in short-chain fatty acids, which are known to have a beneficial effect. The most important short-chain fatty acid is butyrate because of the different beneficial effects on intestinal health and its importance as energy source for the cells. In addition butyrate has also anti-inflammatory properties and the ability to influence the defence barrier and to control the presence of pathogens. Butyrate is mainly produced by bacteria belonging to *Clostridium* cluster I, IV, XIVa and XVI. Their growth and/or activity can be selectively stimulated by non-digestible ingredients or prebiotics. Especially in the chicken, cluster XVI appears to be more important than in other animal species or in human.

The aim of this thesis was to search for a carbohydrate or dietary fibre that would improve broiler performance by inducing a microbial shift in the gastro-intestinal tract. The use of xylo-oligosaccharides (XOS) and cellulose in broiler feed were investigated.

In order to understand the full extent of bacterial diversity in the gut it is essential to characterize and classify the microbial organisms present. Especially for *Clostridium* cluster XVI, there is still a lot of confusion. Therefore in the first experimental study, we classified a new isolate and reclassified three misnamed bacteria all belonging to the butyrate-producing *Clostridium* cluster XVI or *Erysipelotrichaceae* family. The new isolate had 96% 16S rRNA gene sequence similarity with its phylogenetic neighbour *Streptococcus pleomorphus*. Both the new isolate and the misnamed *Streptococcus pleomorphus* were classified in a new genus with the name *Faecalicoccus*. The new species was given the name *Faecalicoccus acidiformans*. In addition, two other phylogenetic neighbours were reclassified, *Eubacterium cylindroides* became *Faecalitalea cylindroides* and *Eubacterium biforme* became

Holdemanella biformis. It was shown that they all produce lactate and butyrate as major fermentation end products in the M2GSC medium. The description of these strains would allow for a number of operational taxonomic units (OTUs) to be classified as one of these new genera with the aid of the ribosomal database project.

In the second experimental study, we demonstrated a positive effect on broiler performance of XOS by decreasing the feed conversion ratio. In addition, with the aid of 16S rRNA sequencing and qPCR, the influence of XOS on the microbiota composition was investigated. Both techniques showed that the number of butyrate-producing *Clostridium* cluster XIVa bacteria were significantly increase in the caeca of the chickens that received XOS. This increase was attributed specifically to the increase of one genus, and within this genus one specific species, *Anaerostipes butyraticus*. In the colon, there was a significant increase of the number of lactate-producing lactobacilli. This increase was attributed to the specific expression of only one species, *Lactobacillus crispatus*. It is already proven that bacteria from *Clostridium* cluster XIVa have the ability to produce butyrate in the presence of lactate. An *in vitro* fermentation assay showed that in the presence of XOS, the lactate production was increased by *Lactobacillus crispatus*. This lactate could then be used by *Anaerostipes butyraticus*, a *Clostridium* cluster XIVa species, for the production of butyrate. This experiment confirmed cross-feeding between lactate-producing and butyrate-producing bacteria and was shown to have a positive effect on broiler performance. Based on the physiological reflux mechanisms colon content is regularly injected into the caeca, thus allowing for the cross-feeding between the XOS degrading lactate producer in the colon and the butyrate-producing, lactate consumer in the caeca.

In the third experimental study, we demonstrated that cellulose supplementation could give a good start for broilers by reducing the feed conversion and increasing the daily gain in the starter phase. Again with the aid of 16S rRNA sequencing and qPCR we looked at the influence of the substrate on the microbiota composition. Although cellulose is traditionally considered to be inert, it did influence the microbiota composition by increasing the genus *Alistipes* in the caeca of broilers receiving the cellulose as compared to the control group. No shifts in the colon microbiota composition were observed. The link between the presence of the genus *Alistipes* and the improved performance of broilers is not clear and further research is necessary.

Around 90% of the bacteria in the gastro-intestinal tract are unknown, indicating that the knowledge of the intestinal microbiota is incomplete. To understand the mechanisms supporting gastro-intestinal health it's important that new genera would be characterized. Beside the isolation and the description of the bacteria, it is even more important to stimulate the beneficial bacteria to improve the broiler health. One of the most important conclusions is that, prebiotics and other feed ingredients can influence the microbial populations of this thesis to the best or to the worst.

SAMENVATTING

Uit wetenschappelijke literatuur is duidelijk dat de darm microbiota direct of indirect fysiologische en pathologische processen beïnvloedt die plaatsvinden zowel in als buiten het verteringsstelsel. De darm microbiota speelt dan ook een belangrijke rol in de gastheer gezondheid en productiviteit. Twee bacteriële phyla, *Firmicutes* en *Bacteroidetes* vormen de grootste microbiële populaties in het spijsverteringsstelsel. De eindproducten van de microbiële fermentatie werken als signaal moleculen en beïnvloeden het metabolisme van de gastheer. In grote lijnen zorgt bacteriële fermentatie van eiwitten voor de vorming van meer schadelijke metabolieten voor de gastheer, terwijl eerder suiker fermentatie resulteert in korte keten vetzuren, met gekende gunstige effecten. Eén van deze korte keten vetzuren is boterzuur, dat de voornaamste energiebron vormt voor de colonocyten. Daarnaast heeft boterzuur anti-inflammatoire eigenschappen en is het in staat de defensie barrière van de darmwand te versterken en de aanwezigheid van pathogenen te onderdrukken. Boterzuur wordt voornamelijk geproduceerd door bacteriën die behoren tot de *Clostridium* cluster I, IV, XIVa en XVI. Hun groei en/of activiteit kan selectief gestimuleerd worden door bepaalde niet-verteerbare voederingrediënten of door gesupplementeerde prebiotica. Voornamelijk bij kippen, lijkt cluster XVI meer van belang te zijn in vergelijking met andere dieren en met de mens.

Het doel van deze thesis was het zoeken naar een voedingscomponent die de zoötechnische prestaties van vleeskippen kan verbeteren door het induceren van een microbiële verschuiving in de darm ten voordele van de boterzuur produceerders. Meer bepaald werd het effect van inmenging van xylo-oligosacchariden (XOS) en cellulose op de darm microbiota en prestaties van vleeskippen onderzocht.

Om de werking van het microbioom in de darm te begrijpen, is het essentieel om de aanwezige microbiële organismen zo goed mogelijk te karakteriseren en te classificeren. Gezien het belang bij de kip en de verwarring in de literatuur wat betreft *Clostridium* cluster XVI, werd hier speciale aandacht aan besteed. In de eerste experimentele studie werd een nieuw isolaat beschreven en geclassificeerd. Daarnaast werden drie verkeerd benoemde bacteriële species herbenoemd. Alle behoren tot de boterzuur-producerende *Clostridium* cluster XVI of *Erysipelotrichaceae* familie. De sequentie van het 16S rRNA gen van het nieuwe isolaat heeft 96% gelijkenis met dat van zijn dichtste fylogenetische buur, *Streptococcus pleomorphus*. Beide, het nieuwe isolaat en de fout benoemde *Streptococcus*

pleomorphus werden geclassificeerd in een nieuw genus met de naam *Faecalicoccus*. Het nieuwe species kreeg de naam *Faecalicoccus acidiformans*. Daarnaast werden twee andere fylogenetische buren herbenoemd, *Eubacterium cylindroides* werd *Faecalitalea cylindroides* en *Eubacterium biforme* werd *Holdemanella biformis*. Er werd aangetoond dat al deze bacteriën melkzuur en boterzuur produceren als belangrijkste fermentatie eind-producten in het M2GSC medium. De beschrijving van deze kiemen zorgt ervoor dat een aantal operationele taxonomische eenheden die bekomen worden door 16S rRNA gensequencing, geclassificeerd kunnen worden als één van deze nieuwe genera met behulp van 'Ribosomal Database Project' (RDP).

In een tweede experimentele studie toonden we het gunstige effect van XOS op de vleeskippen prestaties door een verlaagde voederomzet. Daarnaast werd de invloed van XOS op de microbiota samenstelling onderzocht met behulp van 16S rRNA sequenering en qPCR. Met deze technieken vonden we dat de concentratie aan boterzuur-producerende *Clostridium* cluster XIVa bacteriën significant hoger was in de caeca van kippen die XOS hadden gekregen. Deze verhoging werd toegeschreven aan de verhoging van één genus, met binnenin dit genus één specifiek species, namelijk *Anaerostipes butyraticus*. In het colon, werd een significant hoger aantal melkzuur-producerende lactobacilli gedetecteerd. Deze verhoging werd toegeschreven aan de specifieke verhoging van één enkel species, namelijk *Lactobacillus crispatus*. Het is reeds aangetoond dat bacteriën van *Clostridium* cluster XIVa de mogelijkheid hebben om boterzuur te produceren uitgaande van melkzuur. In een *in vitro* experiment werd aangetoond dat in de aanwezigheid van XOS, de melkzuur productie door *Lactobacillus crispatus* werd verhoogd. XOS hadden geen directe invloed op de metabole activiteit van *Anaerostipes butyraticus*. In co-cultuur echter, werd het melkzuur dat geproduceerd was door *Lactobacillus crispatus,* vervolgens omgezet naar boterzuur door *Anaerostipes butyraticus*. In aanwezigheid van XOS en *Lactobacillus crispatus* produceerde *Anaerostipes butyraticus* dus veel meer boterzuur. Dit experiment bevestigt dat er een uitwisseling mogelijk is tussen melkzuur en boterzuur producerende bacteriën. Deze uitwisseling tussen beide bacteriële groepen kan zorgen voor een positief effect op de prestatie van vleeskippen. De inhoud van het colon wordt regelmatig geïnjecteerd in de caeca door de fysiologische reflux mechanismen waardoor cross-feeding mogelijk istussen XOS afbrekende melkzuur produceerders die zich in het colon bevinden en de boterzuur produceerders-melkzuur verbruikers boterzuur producerende- melkzuur verbruikers die zich in de caeca bevinden.

In een derde experimentele studie tonen we dat het toevoegen van cellulose aan het voeder kan zorgen voor een betere start van de vleeskippen, meer bepaald door het verlagen van de voederomzet en een verhoogde dagelijkse groei tijdens de starter fase. Opnieuw werd met behulp van 16S rRNA sequenering en qPCR gekeken naar de invloed van de experimentele behandeling op de microbiota samenstelling. Er was toename van het *Alistipes* genus in de caeca van de kippen die cellulose hadden gekregen in vergelijking met de controle groep. Er waren geen verschuivingen in de colon microbiota. Ondanks dat cellulose traditioneel als inert wordt beschouwd, toont deze studie aan het dat cellulose de microbiota toch kan beïnvloeden. De link tussen de aanwezigheid van het genus *Alistipes* en een verbeterde prestatie van de kippen is nog niet gekend en moet dus verder onderzocht worden.

Ongeveer 90% van de bacteriën in het spijsverteringsstelsel zijn nog ongekend. Dat toont aan dat de kennis van de darm microbiota nog zeer onvolledig is. Om de mechanismen van de darmgezondheid te begrijpen is het dus van belang dat nieuwe genera en species benoemd en gekarakteriseerd worden. De ongekende bacteriën moeten geïsoleerd worden en functioneel gekarakteriseerd. Op die manier kunnen nieuwe mogelijkheden ontdekt worden om de voordelige bacteriën te stimuleren en zo de darmgezondheid te verbeteren. De take home message van deze thesis is dat het zeker mogelijk is om met prebiotica en andere voedings-ingrediënten, de microbiële populatie in de darm in een gunstige richting te sturen.

ABOUT THE AUTHOR

Celine De Maesschalck werd geboren op 2 november 1986 te Brugge. Na het behalen van het diploma hoger secundair onderwijs aan het Lyceum Hemelsdaele te Brugge richting Latijn-Wiskunde, startte ze in 2004 met de studies Biochemie/Biotechnologie aan de Universiteit Gent. Zij behaalde in 2010 het diploma van Master in de Biochemie en Biotechnologie (optie plant en biomedische biotechnologie).

In 2011 vatte zij bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten van de Faculteit Diergeneeskunde aan de Universiteit Gent haar doctoraatsonderzoek aan, waarin zij het effect van prebiotica op de darmgezondheid van vleeskippen bestudeerde. Dit onderzoek werd gefinancierd door het productschap diervoeding. Verder begeleidde zij verschillende studenten in het behalen van hun masterproef en vervolledigde zij in 2015 het trainingsprogramma van de Doctoral School of Life Science and Medicine van de Universiteit Gent.

Celine De Maesschalck is auteur en co-auteur van verschillende wetenschappelijke publicaties in internationale tijdschriften en gaf verschillende presentaties op meerdere nationale en internationale congressen.

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DANKWOORD

Finally, we did it! Aangezien je een doctoraat niet alleen schrijft, kan het DANKWOORD hier dan ook niet ontbreken.

Eerst en vooral zou ik graag mijn promotoren willen bedanken. Filip, bedankt om 5 jaar geleden in mij te geloven en mij de kans te geven om te mogen starten aan dit doctoraat. Maar zeker ook voor de hulp bij het denkwerk van de verschillende proeven en het boetseren van m'n artikels en andere tekst-gerelateerde projecten. Merci! Prof. Ducatelle, uw deur stond altijd open voor mijn vragen. En als ik nog maar eens vond dat de resultaten niet helemaal waren zoals we gehoopt hadden, dan was u er altijd wel om toch maar weer het positieve te zien in mijn data. Bedankt om mij steeds op weg te helpen met uw suggesties en ideeën, maar ook voor het nalezen van mijn doctoraat. Venessa, Jij hebt ervoor gezorgd dat ik de nodige anaerobe handelingen onder de knie kreeg, toonde mij hoe je het best faecal DNA isoleerde, hoe je het qPCR toestel aan de praat kreeg en nog zoveel meer… Daarnaast ben ik zeker wel 1000^2 keer komen kloppen op je deur, om dan binnen te komen met 'ik heb eens een vraagje…'. En dan het schrijven van dit doctoraat, Merci echt waar voor alle hulp bij het nalezen en verbeteren. Ik weet dat het niet altijd gemakkelijk is geweest voor jou en al zeker niet omdat we beiden geen grote fan zijn van 'het schrijven', maar mede dankzij jouw vele hulp is het dan toch gelukt om alles op papier te krijgen. 1000 keer Merci!

Daarnaast zou ik ook graag Prof. Haesebrouck bedanken omdat hij mij de mogelijkheid heeft gegeven om onderzoek te kunnen uitvoeren in een labo met alle mogelijke middelen en daarnaast ook voor het nalezen en verbeteren van mijn publicaties.

Verder zou ik ook graag de leden van mijn begeleidings-commissie, Prof. Dr. Siska Croubels en Dr. Luc Maertens, bedanken voor het kritisch nalezen in de voorronde van het indienen van mijn doctoraat. Ook de leden van mijn lees- en examencommissie verdienen hier een woordje van dank: Prof. Dr. Dominiek Maes, Prof. Dr. Tom Van de Wiele, Dr. Leon Marchal en Prof. Dr. Geert Huys, bedankt voor jullie nuttige en interessante tips, suggesties en opmerkingen tijdens het afronden van dit doctoraat. Tevens zou ik ook het Productschap Diervoeding willen bedanken voor hun financiële steun en het vertrouwen die ze getoond hebben in dit project. Mijn bijzondere dank gaat daarvoor uit naar Dr. Leon Marchal en Dr. Loek de Lange die hierbij onze directe contactpersonen waren.

Voor dit onderzoek heb ik ook de mogelijkheid gekregen om buiten-huis ervaring te mogen gaan opdoen. Cindy en Margot (LMG) bedankt om mij te leren hoe je het best genomisch DNA bereid met behulp van de pitcher methode, maar ook voor de hulp bij de DNA-DNA hybridisatie en het bepalen van vetzuurpatroon van onze kiemen. Voor het kwantificeren van de korte-keten vetzuren kon ik dan weet terecht bij Siegrid en Jelle. Bedankt om mij in te leiden in de wereld van de HPLC met zijn nodige hinderlagen van foutmeldingen op het toestel tot het goed krijgen van onze pieken om zo tot het juiste resultaat te kunnen komen. Ook Bernard zou ik graag bedanken voor zijn hulp bij het sequeneren. Verder zou ik ook nog graag Annelies (LabMet) bedanken, want ook al hoorde ik niet bij het SBO project toch hielp je steeds waar nodig en het was ook aangenaam om niet helemaal alleen te zijn in Aberdeen op congres. Veel succes met je nieuwe job in Zwitserland.

Maar ook binnen-huis zijn er heel wat mensen die hier hun plaatsje verdienen.

Evy, jij verdient hier meer dan de 1ste plaats! Wie had ooit gedacht dat ik van de planten naar de kippen ging gaan… Na een iets moeilijkere start is het me toch gelukt om te wennen aan al die darmen. Maar nu het bedanken, waar zal ik beginnen? Ik denk dat het iets te veel is om hier allemaal op te noemen dus zeg ik je gewoon bedankt voor alles! Wolf, de enige man in ons midden! Alhoewel de enige… ik denk dat het mannelijk-deeltje-van-onze-hersenpan ervoor kon zorgen dat je niet alleen was, niet? Karen, ik zie je nog toekomen, een beetje verlegen... Maar daar is ondertussen gelukkig niets meer van te merken! Lonneke, mijn thesis-student, ondanks een hobbelig start van jouw thesis-parcours denk ik dat we er toch in geslaagd zijn om er mooie thesis van te maken en dat met misschien zelfs ooit een artikel voor jou. Het was dan ook zeer leuk, dat je bent blijven hangen bij ons. Helaas is je parcours nog steeds wat hobbelig, maar ik ben er zeker van dat je er zal geraken! Evy, Wolf, Karen en Lonneke, bedankt om onze bureau, zo gezellig en leuk te maken!! Sofie G, ook al hebben we nooit echt samen in de les gezeten, een echte onbekende was je toch niet. Ik hoop dat jij de volgende bent die hier mag staan en heel veel succes met het vinden van je droomjob! Karolien, onze dagelijkse bezoeker op de bureau met je duizend vragen waar we helaas niet altijd konden op antwoorden, maar ik ben er zeker van dat jij de antwoorden ondertussen zelf wel gevonden hebt. Sofie K, ook voor jou veel succes met het afwerken van je doctoraat! Gunther en Bonnie, jullie hebben voor de mij de spitst-afgebroken of nee de druk heel hoog gelegd. Maar mocht ik de hulp van jullie allemaal niet gehad hebben bij de vele staalnames en de kippen-weeg-feestjes in het ILVO, dan had ik hier nu niet gestaan. Dus voor jullie allemaal meer-dan-Merci voor alle hulp! Leslie en Wander, bedankt om taxi voor mij te spelen van den

aldi tot op de faculteit toen ik met mijn voet in het gips zat. Want wandelen dat was geen optie! Wie ik hier zeker ook niet mag vergeten te bedanken zijn Christian en Delphine, merci om mijn coupes zo snel te snijden en te kleuren.

Daarnaast moet ik ook Serge, Arlette, Nathalie, Sofie, Connie, Julie, Marleen en Magda bedanken zonder jullie zou onderzoek niet altijd mogelijk zijn, bedankt voor alle hulp! Jo, Gunther en Astra, bedankt om de papieren-rompslomp en mijn bestellingen steeds met een glimlach in orde te brengen! Verder verdienen Pascal, Pudsa, Marc, An, Caroline, Eva, Ellen, Myrthe, Iris, Nele, Maxime, Cheng, Anneleen, Lieze, Gwij, Bram, Elin, Lien, Roel, Chloë, Annemieke, Filip, Tom, Mark, Johan, Michiel, Han, Veronique, Nadia, Marjan, Leen, Marleen, Norbert, Sarah, Joachim, Nathalie, Laura, Justine, Jonas, … ook een woordje van dank voor de leuke momenten in het labo. Merci!

Gedurende deze 4 jaar zijn er ook heel wat mensen vertrokken die hier niet mogen ontbreken: Stefanie, Dorien, Ruth, Sergio, Leen, Beatrice, Sarah, Cyntia, Jordy, Alexander, David, Bregje, Miet, Hanne, Anja^2, Hannah, Evelien, Shoaji, Guangzhi, …

Maar ook mijn nieuwe collega's verdienen hier een woordje van dank. Bedankt om mij een meer-dan-welkom gevoel te geven tijdens mijn eerste weken. We gaan er samen eens een lap opgeven.

Dan zijn we nu aangekomen bij het thuisfront. Dat is zowel het West-vlaamse Brugge als het Oost-vlaamse Gent! Laten we beginnen bij Brugge-die-schone: Loesje, Benjamin en ondertussen ook de kleine Oona-Lou, het is altijd leuk om bij jullie thuis te komen en dat zal in jullie nieuwe huis zeker niet anders zijn. Loesje, ik weet dat ik misschien een klein beetje een work-o-holic ben, maar ik zal mijn best doen om iets meer tijd vrij te maken voor leuke dingen zoals een terrasje of een uitstapje naar de zee, een verre reis is misschien iets te veel van het goede, maar je weet nooit. 1 ding is zeker voor ontspanning kan ik altijd terecht bij jullie, Merci! Veerle en Tine, hoe lang kennen wij elkaar nu al? Lang! Mijn eerste schoolherinneringen zijn deze samen met jullie en ook al zijn we alle 3 een andere weg uitgegaan, we komen elkaar weer steeds tegen en dan is het alsof het nooit niet anders geweest is. Ik hoop dat we nog vele leuke en plezante momenten mogen samen beleven. Charlotte, Basiel en de kleine Leni, bedankt voor de leuke dates of het nu samen koken, een spelletje spelen of iets gaan drinken is… In Gent studeren brengt natuurlijk ook heel wat vrienden met zich mee Lynda, Morgane, Isabel, Kristof, Corinne, … Bedankt voor alle leuke

momenten! Daarnaast mag ik Koen Evy, Fiebe en Finn, zeker ook niet vergeten te bedanken voor de vele leuke momenten van samen naaien, een spelletje spelen, boekjes lezen, iets eten in Fiebe haar keukentje, … Merci! En dan eindigen we in Gent met de 'Bende'! Charlotte, Miek, en Bram: Merci voor de leuke ski/snowboard-vakanties, de gezellige oud-nieuw avond met de kids en ook de vele etentjes! It's time to go crazy! Op naar onze vele weekendjes in d'ardennen, de champagnes-streek en waar gingen we nog allemaal naar toe? Bram, jij verdient hier nog een extra woordje van dank: mijn beste vriend! Zonder jou had ik hier waarschijnlijk al helemaal niet gestaan, want dat Master-diploma heb ik toch ook deels te danken aan jouw cursus met je super-zalig-code-geschrift! Altijd bereid om eentje te gaan drinken en te luisteren naar mijn geklaag en veel gezaag, maar ook steeds paraat voor een klim-avontuur! Ik hoop dat we ondanks al onze vervelende kwaaltjes toch nog eens die rotsen mogen gaan trotseren binnen of zelfs buiten België. Bij deze Merci!

De VIPS komen meestal als laatste aanbod en dat is ook hier niet anders: papa, mama, Camille, Marie + Thomas (joepie, ik heb eindelijk een broer ☺)! Ik zou jullie graag bedanken voor de onvoorwaardelijke steun en omdat jullie steeds zijn blijven geloven in mij! Papa, ik denk dat er niemand meer gevraagd heeft: 'of er nu al een datum was, zodat je die toch wel zeker kon vastleggen in je agenda?' Wel die datum is er eindelijk, hij stond toch in je agenda é? Bedankt voor alles!!

En als laatste kan ik enkel nog zeggen: Merci aan iedereen die komen luisteren is en dan nu Santé, laten we er eentje (of meer) op klinken!