STUDY OF GASTROINTESTINAL COMMUNITIES OF COWS AND PIGS BY METAGENOMICS, WITH THE FOCUS ON METHANE EMISSIONS AND ANTIBIOTIC RESISTANCE

Thijs DE MULDER

Promotors:

Prof. dr. ir. Tom Van de Wiele

Dr. Karen Goossens

Dr. Geertrui Rasschaert

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Applied Biological Sciences

Proefschrift voorgedragen tot het bekomen van de graad van Doctor in de Bio-ingenieurswetenschappen

Promotors

Prof dr. ir. Tom VAN DE WIELE

Center for Microbial Ecology and Technology, Fac. of Bioscience Engineering, UGent

dr. Karen GOOSSENS

Animal Science Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO)

dr. Geertrui RASSCHAERT

Technology and Food Science Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO)

Members examination committee

Chairman of the Examination committee:

Prof. dr. ir. John VAN CAMP
Dep. of Food Safety and Food Quality, Fac. of Bioscience Engineering, UGent

Prof. dr. ir. Veerle FIEVEZ
Dep. of Animal Production, Fac. of Bioscience Engineering, UGent

Prof. dr. ir. Stefaan DE SMET
Dep. of Animal Production, Fac. of Bioscience Engineering, UGent

Prof. dr. Marc HEYNDRICKX
Technology and Food Science Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO)

Prof. dr. Jeroen DEWULF
Veterinary Epidemiology Unit, Dep. of Reproduction, Obstetrics and Herd Health, Fac. Of Veterinary Medicine, UGent

Dr. Diego MORGAVI
UMR Herbivores, Centre INRA (France)

Dean: **Prof. dr. ir. Marc VAN MEIRVENNE**

Rector: **Prof. dr. Rik VAN DE WALLE**

Titel van het doctoraat in het Nederlands:

De studie van de gastro-intestinale microbiële gemeenschappen in runderen en varkens door middel van metagenomics, met oog op methaan emissie en antibiotica resistentie

To refer to this thesis:

De Mulder, T. (2016) Study of gastrointestinal communities of cows and pigs by metagenomics, with the focus on methane emissions and antibiotic resistance. Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) of Applied Biological Sciences. Faculty of Bioscience Engineering, Ghent University.

ISBN:

Table of contents

TABLE OF CONTENTS

TABLE OF CONTENTS
ABBREVIATION LIST

CHAPTER 1
INTRODUCTION

CHAPTER 2

EXPLORING THE METHANOGEN AND BACTERIAL COMMUNITIES OF RUMEN ENVIRONMENTS: SOLID ADHERENT, FLUID AND EPIMURAL

CHAPTER 3

IMPACT OF BREED ON THE RUMEN MICROBIAL COMMUNITY COMPOSITION AND METHANE EMISSSIONS OF HOLSTEIN FRIESIAN AND BELGIAN BLUE HEIFERS

CHAPTER 4

HOST INFLUENCES AND DYNAMIC FLUCTUATIONS OF THE BACTERIAL AND METHANOGEN COMMUNITY FOLLOWING A COMPLETE RUMEN CONTENT EXCHANGE

CHAPTER 5

THE EFFECT OF RESIDUES OF DOXYCYCLINE, DUE TO CROSS-CONTAMINATION OF FEED, ON THE MICROBIAL COMMUNITY IN PIG'S FECES

CHAPTER 6

IMPACT OF SUBTHERAPEUTIC CONCENTRATIONS OF DOXYCYCLINE ON THE MICROBIAL ECOSYSTEM IN AN IN VITRO MODEL OF THE PIG'S CECUM

CHAPTER 7

GENERAL DISCUSSION AND PERSPECTIVES

Abbreviation list

ABBREVIATION LIST

A	
Ad	Sample of the eluted solid-adherent fraction attached to rumen fibers
AMR	Antimicrobial resistance
ARISA	Automated ribosomal intergenic spacer analysis
B	
BCC	Bacterial community composition
BCFA	Brached chain fatty acids
BW	Body weight (kg)
C	
CH_4	Methane
CO_2	Carbon dioxide
CRL	Crude (unstrained) rumen liquid
CSS	Cumulative sums scaling
D	
DGGE	Denaturing gradient gel electrophoresis
DMI	Dry matter intake
DMBB	Double-muscled Belgian Blue (beef cattle)
DOX	Doxycycline
DOX-h	Doxycycline hyclate
DPI	Protein digested in the intestine
DWG	Daily weight gain
E	
Ep	Sample of the epimural fraction in the rumen
ECOFF	Epidemiological cutoff value for resistance
F	
F	Oral bioavailability
FAO	Food and Agriculture Organization (United Nations)
FCR	Feed conversion ratio
FISH-CLSM	Fluorescent in situ hybridization & confocal laser scanning microscopy
G	
GFE	Gross feed efficiency
GIT	Gastro-intestinal tract
H	
HF	Holstein-Friesian (dairy cattle)
HTS	High throughput sequencing
J	
JE	Jersey (dairy cattle)
L	
LAB	Lactic acid producing bacteria
LOD	Limit of detection
Lq	Strained rumen liquid

M

MIC	Minimum inhibitory concentration

N

NDF	Neutral detergent fiber
NE	Net energy
NGS	Next Generation Sequencing
NMDS	Non-metric multidimensional scaling

O

OTU	Operational taxonomic unit

R

RCC	Rumen Cluster C
RDP	Ribosomal Database Project (aligned and annotated rRNA gene sequence data)
RDPB	Rumen degradable protein balance
RFLP	Restriction Fragment Length Polymorphism
RIM-DB	Rumen and Intestinal Methanogens-database (annotated 16S sequences of intestinal methanogens)

S

S	Sample of the rumen solid phase (digested fibers)
SCFA	Short chain fatty acids
SEM	Scanning electron microscopy
SRA	Sequence read archive

T

tet	Tetracycline resistance (gene)

V

VFA	Volatile fatty acids

W

WGS	Whole genome shotgun (sequencing)

Chapter 1

Introduction

CHAPTER 1 INTRODUCTION

1.1 GENERAL INTRODUCTION

The world is facing a rising demand for meat production to satisfy the growing human population and the improved living standards in developing countries. Since 1961, the first year with FAO data, the global meat production has grown from 71 million tons to 317 million tons in 2014, corresponding to an average yearly increase of 2.8%. Likewise, the global milk production has increased from 344 million tons in 1961 to 802 million tons in 2014 (Figure 1.1) [1]. Undoubtedly, these yearly increases will progress further in the future to meet increasing demands. The requirement of cheap and high throughput production of meat and milk on diminishing agricultural space has induced the intensifying of livestock production which contributed to the environmental impact of agriculture. The scientific community together with governmental institutes are now facing the immense challenge to increase livestock production yield, while decreasing its correlated environmental impact.

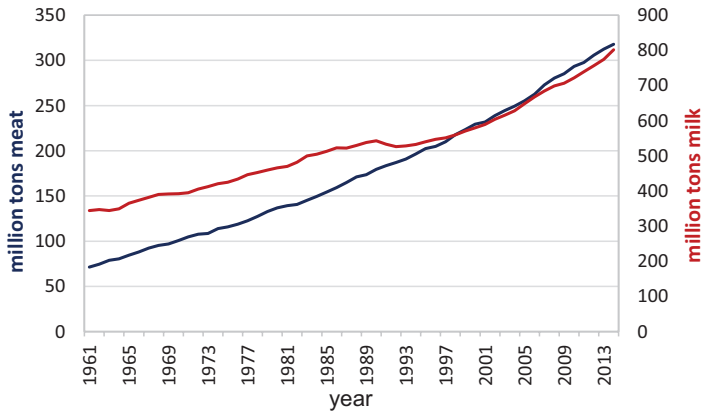


Figure 1.1 The global meat (**blue**) and milk (**red**) production per year (1961-2014)

The focus of this PhD thesis was the investigation of the microbial communities in the rumen of cattle and in the intestinal tract of pigs, by metagenomics techniques.

Ruminant animals (sheep, goat, cattle) rely on vast and complex microbial populations in the rumen to ferment fibrous feed to accessible nutrients such as volatile

fatty acids, vitamins and microbial protein which can be absorbed by the rumen epithelium or further in the gastrointestinal tract. Methanoarchaea occupy a central role in this rumen fermentation process by converting dissolved CO_2 and H_2 gasses, both by-products of anaerobic fermentation, to methane. This methane, a potent greenhouse gas, is released into the atmosphere via frequent belching. According to FAO estimates, enteric fermentation of cattle was responsible for an emission of 72.5 billion kg CH_4 in 2014, which corresponds to 1 522 billion kg CO2-eq. and 29% of the global emissions of CO2-eq. from agriculture [1]. In Belgium, the agricultural sector produced 6.7 million kg CO2-eq., which contributed 8% to the total Belgian greenhouse gas emissions in 2014. Enteric fermentation was responsible for 35% of the agricultural greenhouse emissions [2].

Rising demand for more and cheaper meat and milk pressured the farmers to increase production at lower costs. Characteristic to intensive livestock production is the dense housing of animals, allowing infections to spread quickly. As a consequence, antibiotic use in livestock farming has increased worldwide. Farmers turn to antibiotics to treat individual animals or entire herds after symptoms have been identified, to prevent the outbreak of diseases or as growth promoting agent, although the latter is forbidden in certain countries. Medicated feed and drinking water can introduce a diverse range of antibiotics in the gastrointestinal tract of animals, where they provide the opportunity to resistant bacteria to proliferate. Resistant bacteria are shed out with the feces and can contaminate meat, transfer to the animal caretaker or spread in the environment when the fecal material is used as fertilizer. In this manner, intensive agriculture contributes to a topical and major public health threat: the spread of antimicrobial resistance and the rising occurrence of (multi)drug-resistant pathogens. According to the European Centre for Disease prevention and Control (ECDC); each year approximately 400 000 people become infected with multidrug resistant bacteria in the EU, of which around 25 000 people die each year as a result of these infections. Limiting the antibiotic (mis)use in livestock production may lower the exposure of animals and humans to resistant bacteria. Governmental restrictions and regulations, like the prohibition of antibiotics as growth promoting agent and the requirement for veterinary prescription for antibiotic treatment are already one step into the right direction. Unfortunately, cross-contamination of antimicrobial compounds from medicated feed to non-medicated feed still cause animals to be unintentionally exposed to antibiotics.

1.2 CHARACTERIZING THE RUMEN MICROBIAL ECOSYSTEM

1.2.1 The rumen microbial ecosystem

1.2.1.1 The rumen optimized for microbial fermentation

Ruminants, including cows, sheep, goat but also giraffes, deer, yaks and antelopes depend on the rumen to convert fibrous feed into readily available energy sources for the host. These fibrous feeds (grass, hay, silage) consist primarily out of cellulose, a major constituent of plant cell walls and as such the most common organic polymer on earth [3]. Mammals are generally unable to digest (hemi)cellulose and outsource this task to microorganisms. To this end, ruminant animals accommodate an extensive microbial community in a specialized intestinal compartment: the rumen.

The rumen is (functionally) the first forestomach of a four-compartment stomach and is optimized for microbial growth and fermentation of fibrous feed. The rumen contains roughly three phases: a gas layer, a fibrous mat of freshly ingested feed and rumen fluid containing degraded and fragmented fibers. These phases are frequently mixed by 1-3 contractions per minute to bring the newly digested feed into contact with the rumen fluid. The capacity of an adult dairy cow's rumen is around 80-100 liters, resulting in a residence time of solid feed ranging from 25 to 57 h, depending on the diet type and fiber size [4,5]. The prolonged retention of feed is necessary to give microorganisms sufficient time to attach and digest the fibers. Fiber digestion is enhanced by optimal rumen ambient conditions, creating an optimal environment for bacterial growth and fermentation: a regulated temperature (38-40°C), an oxidation-reduction potential (Eh) of around -0.35 to -0.42 V, a near neutral pH (roughly 6.8) and anaerobic conditions [6]. These rumen conditions are, to a large extent, dependent on the diet type. More fibrous nutrition decreases the passage rate and also increases chewing time and rumination, which in turn will increase salivary production and the rumen pH. On the other hand, diet type can also influence VFA production which again influences rumen pH [7].

1.2.1.2 The rumen microbiome

The rumen harbors a complex microbial consortium of hundreds of different species, represented by billions of microorganisms: the symbiotic rumen microbiome. A dense

bacterial population contributes to 40-90% of the microbial biomass, with numbers ranging from 10^9 to 10^{11} species per gram rumen content [8,9]. The domain of *Archaea* is exclusively represented by methanogens who are present in numbers ranging from 10^5-10^8 species per gram [9]. Further the rumen accommodates 10^4-10^6 protozoa and 10^2-10^4 fungi per gram [8,9] which, despite their relatively low abundances, still make up a large part of the biomass due to their larger size.

1.2.1.3 Rumen bacteria

The rumen bacteria accommodates a variety of bacterial species, predominantly Gram-negative, that are essential for optimal rumen functioning. Classic knowledge about the rumen bacterial community was originally based on culture-dependent studies. Using cultivation, researchers were able to identify and characterize a handful of dominant rumen colonizers (Table 1.1). However these isolated species only represent the tip of the iceberg as the majority of the bacterial population remained anonymous by their inability to be cultivated. Sequencing techniques and other novel culture-independent molecular techniques have refined our view of the rumen bacterial community [10]. In particular, the rumen microbial community demonstrates a high bacterial richness and redundancy (multiple species can occupy the same niche). The functional redundancy coupled with the metabolic flexibility of most bacteria (most species can metabolize a range of nutrients) offers the community a high degree of resilience against perturbations [11].

The functional redundancy of the bacterial community implies that dominant rumen bacteria need to outcompete others to maintain a niche. Primary functional niches are created by the influx of feed containing cellulose, hemicellulose (i.a. xylans) and pectins as major constituents of plant cell walls, starch (stored as energy reserve in amyloplasts in plant cells or corn and grass grains) and protein, nucleic acids and lipids provided by the cytoplasm of plant cells. These substrates are fermented with the production of metabolic end-products (i.e. lactate, succinate, acetate, butyrate and propionate), breakdown products (i.e. dextrins) and gases (CO_2 and H_2), generating secondary functional niches for bacteria able to consume these metabolic by-products. Oxygen is introduced into the rumen with feed and water ingestion and is rapidly consumed by the few facultative anaerobic and aerobic bacteria in the rumen.

Rumen bacteria can be subcategorized into functional groups according to their metabolic capacities: cellulolytic, amylolytic, xylanolytic, pectinolytic, proteolytic, lipolytic, ureolytic, dextrin -, succinate -or lactate utilizers and H_2/CO_2 metabolizers. These

Table 1.1 Metabolic capacity and fermentative properties of bacterial species isolated from the rumen.

Substrate	Substrate fermenting bacteria	Possible metabolic end-products
Cellulose	**Cellulolytic**	
Plant cell wall component;	*Ruminococcus flavefaciens* [12]	Acetate, succinate formate, H_2, CO_2 [12]
linear and long chains of anhydrous glucose; crystalline and resistant to hydrolysis	*Ruminococcus albus* [13]	CO_2, acetate, formate, H_2 [13]
	Fibrobacter succinogenes	Succinate, acetate, formate [15], no hydrogen production [16]
	(*Butyrivibrio fibrisolvens*) [14]	
	(*Clostridium lochheadii*)	
Hemicellulose (incl. xylan, xyloglucan, etc.)	**Xylanolytic**	
Plant cell wall component;	*Prevotella ruminicola* [17]	Acetate, propionate, succinate (H_2, CO_2) [21]
Short heteropolysaccharide chains with amorphous structure of little strength	*Prevotella bryantii* [18]	Glycogen (intracellular storage), acetate, succinate [22]
	Butyrivibrio fibrisolvens [19]	Butyrate, lactate, formate, CO_2 [23]
	Butyrivibrio proteoclasticus [20]	Formate, butyrate, acetate, H_2 [24]
Pectin	**Pectinolytic**	
Plant cell wall component;	*Prevotella ruminicola* [25]	
Heteropolysaccharide	*Butyrivibrio fibrisolvens* [25]	
	Lachnospira multiparus [26]	Acetate, formate, lactate, H_2 [26]
	Succinivibrio dextrinosolvens [27]	Succinate, acetate, formate, lactate [29]
	Streptococcus bovis [28]	Lactate, acetate, formate, CO_2 [30]
Starch	**Amylolytic**	
Polymer of glucose connected by glycosidic bonds	*Prevotella ruminicola*, *brevis*, *albensis*, *bryantii* [31]	
	Butyrivibrio fibrisolvens [32]	
	Ruminobacter amylophilus [32]	Acetate, formate, succinate [34]
	Succinomonas amylolytica [33]	Acetate, propionate, succinate [35]
	Succinivibrio dextrinosolvens [29]	
	Streptococcus bovis [32]	
Protein / peptides	**Proteolytic**	
Cytoplasmic content of plant cells or microbial origin	*Prevotella ruminicola* [36]	Ac./prop./succ. and NH_4^+ (also used a N-source) [39], isobutyrate, isovalerate [40]
	Ruminobacter amylophilus [37]	
	Butyrivibrio fibrisolvens [38]	
Cellodextrin (dextrin) / cellobiose	*Succinivibrio dextrinosolvens* [29]	
Short glucose polymers resulting from cellulolysis or starch hydrolysis	*Selenomonas ruminantium* [41]	Acetate, propionate, succinate
	Butyrivibrio fibrisolvens [41]	
	Ruminococcus albus [41]	
	Ruminococcus flavefaciens [41]	
	Treponema bryantii [42]	Succinate, acetate, formate [42]
Lactate	**lactilytic**	
Fermentation end-products	*Megasphaera elsdenii* [43]	Acetate, propionate, butyrate, H_2 [43]
	Veillonella alcalescens [44]	Propionate, acetate, H_2/CO_2
	Selenomonas ruminantium	
Succinate	*Selenomonas ruminantium* [45]	
Fermentation end-products	*Veillonella parvula* [46]	
	Succiniclasticum ruminis [47]	

divisions are ambiguous as many bacteria display phenotypic diversity and can often utilize a variety of substrates (Table 1.1). More commonly, bacteria are classified by taxonomy. The taxonomic composition of the rumen bacterial community at phylum level is dominated by the *Bacteroidetes* and *Firmicutes*, representing 80% of the community and complemented with lower abundant *Proteobacteria*, *Actinobacteria*, *Tenericutes*, *Fibrobacteria*, *Spirochaetes* and *Cyanobacteria* [10,48]. At genus level, the *Prevotella* (*Bacteroidetes*) is the most prevalent and can account for 30-60% of the bacterial 16S rDNA in the rumen, depending on the diet [10,49,50]. To our knowledge only four *Prevotella* species have yet been isolated from the rumen: *P. ruminicola*, *P. brevis*, *P. albensis* and *P. bryantii* [31]. The isolated *Prevotella* species display genetic and functional divergence [51], reflected in a broad polysaccharide degrading potential [52]. Presumably, *Prevotella* owe their dominance in the competitive rumen ecosystem thanks to their wide range of functional abilities. Similar to *Prevotella*, *Ruminococcus flavefaciens*, *R. albus* (*Firmicutes*) and *Fibrobacter succinogenes* (*Fibrobacteria*) are also consistently present in the rumen ecosystem although they do not have a broad metabolic capacity. These species owe their success solely to their ability to attach and digest cellulose [53] and are therefore recognized as the main cellulolytic bacteria in the rumen. Other uncultivable bacteria may also contribute to cellulose digestion. Naas et al. (2014) reconstructed the genome of an uncultured rumen *Bacteroidetes* species and discovered preliminary evidence of the polysaccharide utilization locus (PUL)-catalyzed conversion of cellulose [54]. Cellulolytic bacteria are the first colonizers of freshly ingested fibers and prime the way for secondary colonizers feeding on the metabolic end-products and degradation products. A perfect illustration of the extensive microbial interactions is the cross-feeding of succinate. Pure cultures of many rumen isolates are known to produce succinate as major end-product of carbohydrate fermentation, including *P. ruminicola* [21,25], *Ruminobacter amylophilus* [55], *F. succinogenes* [56], *R. flavefaciens* [56], *Succinivibrio dextrinosolvens* [29], *Succinomonas amylolytica* [35] and some species from the *Spirochaetes* [57]. Although these prevalent rumen bacteria can produce succinate, it does not accumulate in the rumen but instead serve as an intermediate of fermentation. Succinate-decarboxylating bacteria can rapidly convert succinate to propionate [58]. Using co-culture experiments, Sawanon et al. (2006) determined that cellulolytic activity of *R. flavefaciens* was enhanced by the presence of *Selenomonas ruminantium*, a succinate-consuming bacterium. In monocultures of *R. flavefaciens*, succinate accumulated in the medium while propionate was the main end-product in co-culture [59]. Other co-culture experiments have further confirmed the

importance of cross-feeding between cellulolytic and non-cellulolytic bacteria. The cellodextrin efflux from cellulose digestion can attract and support the growth of non-adherent bacteria. *Treponema bryantii*, a saccharolytic spirochete, was detected during the isolation of cellulolytic bacteria from the rumen [42]. Electron microscopy suggested that *T. bryantii* associates with plant cell mass but consumes the cellodextrin that becomes available during cellulose digestion by neighboring cellulolytic bacteria [42,60]. Co-cultivation of cellulolytic rumen strains with *P. ruminicola* also improved cellulose digestion [61,62]. It would seem that cellulolytic bacteria depend on synergistic interactions with non-cellulolytic bacteria to optimize cellulose digestion. Mutually beneficial interactions also exist amongst non-cellulolytic bacteria. High-starch diets allow the proliferation of *Streptococcus bovis*, which produces lactate as major end-product of starch fermentation. Lactate, more than other VFAs, is highly acidic and when given the chance to accumulate, will rapidly reduce the pH and cause rumen acidosis [63]. Under normal dietary circumstances, lactate does not accumulate but is fermented by *Megasphaera elsdenii* [43,64], *Veillonella alcalescens* [65] and *Selenomonas ruminantium* [66].

A dominant member of the rumen microbial community has to meet a few requirements: the growth conditions of a bacterial species must be adapted to the ambient conditions of the rumen. The species must also outcompete other species competing for the same resources in the ecosystem. Next to synergetic relationships, interspecific competition can be regarded as a second common type of interaction between species. There are two major forms of competition: interference competition and exploitative competition [67].

Interference competition occurs when a species directly alters the resource-attaining behavior of other species [67]. An example of this interaction is found amongst the cellulolytic bacteria. Digestion of cellulose depends on adhesion of cellulolytic species to the substrate. The affinity to cellulose, the rate of adherence and preference for adhesion sites will determine the outcome of competition between *F. succinogenes*, *R. flavefaciens* and *R. albus*. *In vitro* experiments with radiolabeled ($^{14}C/^{3}H$) strains indicated that *R. albus* can interfere with the adhesion of *R. flavefaciens* and *F. succinogenes* [68], providing a selective advantage for *R. albus* under cellulose-limited conditions. However during cultivation in cellulose-excess batch culture, the cellulolytic strains did not need to compete for substrate and were present in almost equal population sizes [69]. Another example of interference competition amongst rumen bacteria is the production of bacteriocins. These are antimicrobial peptides produced by bacteria to inhibit growth of (non-)related

strains/species while the producing strain will protect themselves with proteins conferring resistance or inherent insensitivity [70]. Many rumen isolates were found capable of producing bacteriocins [71,72] which could give them an advantage when occupying a niche by inhibiting growth of competing bacteria.

Exploitation competition occurs when individuals interact indirectly via a shared resource. One species will more efficiently consume and reduce a limiting resource, thus depleting the availability for other species [67]. For example, the genus of *Prevotella* owes their dominance to their nutritional versatility and their rapid growth rate. Many carbohydrate monomers and polymers, as well as amino acids and peptides can support their growth and depending on the substrate, *P. ruminicola* has a doubling time of 46 min (glucose) to 1.5 h (maltose) [22].

Culture-independent analysis of the rumen bacteria, especially using recently developed next generation sequencing (NGS) techniques help to study the rumen microbial community as a whole and provide a better picture of the richness and diversity of the rumen bacterial community. Based on 16S based metabarcoding experiments described in literature, the bacterial richness within a rumen ecosystem was reported between 1000-2000 OTUs [73–76]. Differences between richness amongst different studies can be accounted to biological factors (differences in physiological state, breed, diet composition) and/or technical factors (DNA extraction method, primer choice, sequencing platform and sequencing depth). It is also important to realize that these sequence-based richness estimations are based on certain technical assumptions, most noteworthy the ≥97% similarity to cluster sequenced reads to one OTU. The high richness observed with NGS emphasizes the gap between the actual number of species in the rumen and the number of cultivated representatives. Creevey et al. (2014) carried out a meta-analysis using information from culture collections and sequence databases and linked this to seven published studies of the rumen microbiome. By concatenating the sequences obtained from these seven rumen sequencing studies with the annotated sequences of RDP into a single dataset, the authors designed a representative phylogenetic tree of 2405 rumen bacterial OTUs (Figure 1.2). Analysis of these OTUs confirms that species from the *Prevotellaceae* family dominate the rumen, followed by the families *Lachnospiraceae* and *Ruminococcaceae* [77]. Many other families complete the bacterial community. The rumen bacterial community in steers spanned 24 phyla, 48 classes, 89 orders, 173 families and 317 genera (Illumina MiSeq, 16S V1-V3, Greengenes database) [78]. In samples collected

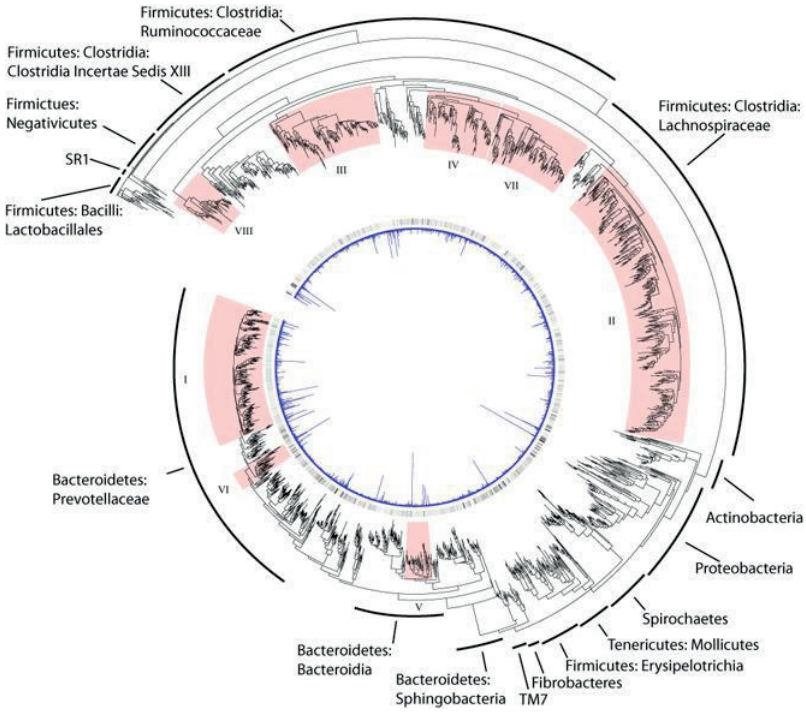


Figure 1.2 Inverted circular phylogenetic tree of 2405 rumen bacterial OTUs. The blue graph indicates the average scaled proportion of each OTU across seven analyzed datasets. The surrounding gray-gradient represents the prevalence of each OTU in the datasets (dark = most prevalent). The major groups of bacteria are identified. The most abundant clades in the rumen are marked red and numbered I to VIII in order of abundance [77].

from the rumen of Asian yaks 21 phyla, 35 classes, 75 families and 112 genera were detected (with Illumina MiSeq, 16S V3-V4, RDP classifier) [79] while in the rumen of Canadian cervids a total of 13 phyla, 141 families and 327 genera were identified (454 pyrosequencing, 16S V1-V3, Silva database) [80]. In contrast, cultivated rumen representatives only include bacteria from only 88 genera belonging to nine phyla [77]. Notably, these metabarcoding studies made use of different databases, most noteworthy: ARB-Silva, Greengenes and RDP, containing fully aligned (quality and chimera checked) 16S rRNA gene sequences of known species. The identification of OTUs in a metabarcoding experiment are therefore dependent on the extensiveness of the databases and thus still depend on the identification and characterization of novel species through cultivation. An international consortium of research institutes joined forces in the Hungate1000 project to gain a better understanding of the function of main rumen bacteria.

This project aimed to produce a reference set of rumen microbial genome sequences by sequencing the whole genomes of cultivated rumen bacteria and archaea [81].

1.2.1.4 Fermentation by-products

The main end-products of rumen fermentation are microbial biomass and volatile fatty acids (VFAs), which serves as nutrients for the host. Roughly, rumen fermentation can be divided into proteolysis and carbohydrate hydrolysis. The fibrous diets provide carbohydrates as substrate for anaerobic hydrolysis: a consortia of microbial enzymes (hydrolases) acts serially to decompose these complex polysaccharides to shorter oligosaccharides or monosaccharides, these simple sugars are further used in fermentation processes to produce acids and gases. For example, endo-cellulases cleave internal bonds at amorphous sites in crystalline cellulose to form separate cellulose chains. Exo-cellulases can then cleave two to four units of the exposed cellulose chains, thereby generating di -or tetrasaccharides. β-glucosidase hydrolyze the exo-cellulase products to soluble glucose molecules which are converted to pyruvate in the glycolysis pathway and further converted to a wide range of volatile fatty acids [82–84]. The end-products of carbohydrate hydrolysis include main short chain fatty acids (SCFA) acetate, butyrate and propionate. Also lactate and succinate are produced by some members of the rumen microbiome. However, these components do not accumulate but are rapidly converted to SCFAs by cross-feeding bacteria. Besides lactate, succinate and SCFAs, also CO_2 and H_2 are major end-products of anaerobic fermentation of carbohydrates. These gases are converted to methane in a process called methanogenesis to avoid accumulation of H_2 (Figure 1.3). The type of produced end-products depends on the metabolic pathways of the species (Table 1.1) and (as always) the diet composition.

Theoretical fermentation balances, although simplified and based on assumptions, permit the calculation of VFA distributions in the rumen. The molar fermentation balance by Wolin (1960) gives the following molar distributions:

$$57.5(C_6H_{12}O_6) \rightarrow 65\ \text{Acetate} + 20\ \text{Propionate} + 15\ \text{Butyrate} + 60\ CO_2 + 35\ CH_4 + 25\ H_2O$$

[85]

Protein is provided to the rumen in the cytoplasmic content of grass, silage and concentrate (beet pulp, soy meal, etc.) and fuels the proteolysis activities of rumen microbes. Many rumen bacteria have proteolytic activity and hydrolyze rumen degradable proteins (RDP) in the feed to small peptide and amino acids using extracellular proteases [86]. Free amino

acids are taken up by bacteria and the remainder is deaminated to ammonia and a C-skeleton, which is further converted to isobutyrate and isovalerate. The ammonia is used by other microorganisms as nitrogen source for amino acid synthesis [87]. The crude protein content from a fibrous diet is not always adequate and nitrogenous compounds (for example, a protein-rich concentrate) are required to cover the needs. Many bacteria can also synthesize microbial protein from non-protein sources such as urea, which can be included in a concentrate as feed additive [88]. Excess ammonia is absorbed across the rumen wall and detoxified back to urea in the liver. Between 40-80% of the urea-N synthesis in the liver is returned back to the gut and mainly the rumen (i.e. urea recycling) [89–91], where it is converted back to aqueous ammonia for further anabolic use [92]. Remaining urea is excreted with the urine [92,93]. Other metabolic end-products are produced to a much lesser extend as compared to the main metabolites (VFAs, ammonia and CH_4), amongst others indolic compounds [94], amines [95] and sulfides [96].

1.2.1.5 Rumen methanogens and methane production

The domain of *Archaea* comprises < 3% of the rumen prokaryotic population [97,98] and is represented exclusively by hydrogenotrophic methanogens from the phylum of *Euryarchaeota*. Archaea are distinct from bacteria by the lack of peptidoglycan in the cell walls and methanogens specifically possess some unique enzymes and coenzymes. The fluorescent coenzyme F_{420} provides methanogens with a characteristic blue-green fluorescence [99] and coenzyme M is an indispensable cofactor required for methyl-transfer in the methanogenesis pathway [100]. Unlike bacteria, methanogens do not depend on high richness and diversity to maintain a stable and resilient community. Typically, the methanogen communities in the rumen ecosystem are limited in both absolute abundance and taxonomic diversity. To our knowledge, methanogens detected or isolated from the rumen belong to four families and eight genera (Table 1.2). *Methanobrevibacter* spp. make up between 60-90% of the methanogen community and species from the *Mbb. ruminantium* clade and the *Mbb. gottschalkii* clade are the most prominent [101–103]. The second largest fraction of methanogens in the rumen are affiliated with *Thermoplasmata*. This clade of uncultured methanogens have been reclassified and renamed several times in the last decade. The nomenclature that we will use in this doctoral thesis is: *Methanomassiliicoccales* - *Methanomassiliicoccaceae*. This order and family were proposed by Lino et al. (2013) as a novel methanogenic lineage in the class of *Thermoplasmata* [104] and is also used in the Rumen and Intestinal Methanogen database

(RIM-DB) [105]. Alternatively, this clade is also indicated as "Rumen Cluster C" (RCC) [87,88] and *Thermoplasmatales* (*Thermoplasmataceae*) [87]. Together with *Methanosphaera* spp., the *Methanobrevibacter* and the genera in the family of *Methanomassiliicoccaceae* represent a large majority (up to 90-98%) of the methanogen community [102,108–110]. Other genera (Table 1.2) are also present but do not represent major players in the rumen and are not consistently detected. Methanogens can occur free-living in the rumen fluid, in association with solid adherent biofilms, attached to the epithelium and associated as epi -or endosymbionts of protozoa. These different environments pass the rumen at different rates and could select for habitat-specific methanogen species, which might explain the phylogenic diversity of the methanogen community [98].

Table 1.2 Phylogenetic distribution of methanogens isolated from the rumen of cows and sheep

Class	order	family	genus
Methanobacteria	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium* *Methanobrevibacter* *Methanosphaera*
Methanomicrobia	*Methanosarcinales*	*Methanosarcinaceae*	*Methanimicrococcus* *Methanosarcina*
	Methanomicrobiales	*Methanomicrobiaceae*	*Methanomicrobium* *Methanoculleus*
Thermoplasmata	*Methanomassiliicoccales*	*Methanomassiliicoccaceae*	

Ingested feed enters the rumen and is fermented by a mixed culture of bacteria with the production of VFAs, NH_4^+, CO_2 and H_2. Hydrogen gas is locally produced by active bacteria and, due to its non-polar character, can freely pass through microbial membranes striving towards an intra- and extracellular equilibrium. Accumulation of H_2 will increase the partial pressure (P_{H2}) which in turn causes feedback inhibition of fermentation pathways [111]. Consequently, despite methanogens only make up a small part of the rumen microbial population, they occupy a significant role in its function. Rapid H_2 removal by methanogenesis (or alternatively by propionate synthesis and reductive acetogenesis) leads to more favorable conditions for fermentation and will increase VFA productions. Most species of methanogens, especially the dominant rumen representatives, can grow on H_2/CO_2 (hydrogenotrophic) and often formate as sole substrate of methanogenesis. Some species can also grow on methyl groups or acetate (although not to a significant extent)

[98] (Figure 1.3). The major end product of methanogenesis is methane, which accumulates in the headspace of the rumen and is released by eructation. Methane emissions are thus an inevitable outcome of enteric fermentation. Enteric methane emissions by dairy cows is in the range of about 300 and 450 g CH_4 day^{-1} and vary with feed intake, diet composition and milk yield [112–114]. Even under conditions of equal diet composition and feed intake, the methane production between cows can vary, suggesting also an influence of the host (genotype, physiological stage, life history, age) on methanogenesis [115,116].

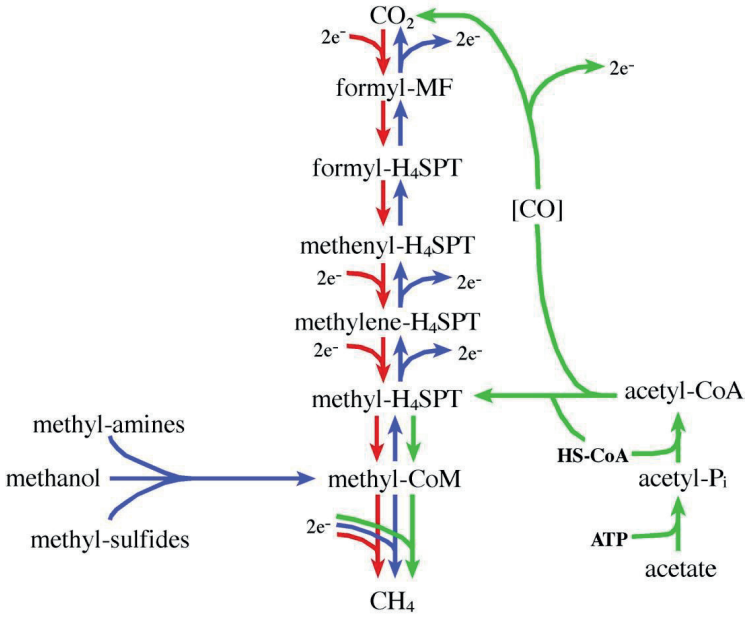


Figure 1.3 Simplified overview of the different paths of methanogenesis. The hydrogenotrophic (red), the methylotrophic (blue), and the aceticlastic (green) pathway [117].

1.2.2 Rumen environments

The rumen microbiome is a collection of well-organized consortia of organisms proliferating in three rumen environments: free-living in the rumen fluid, associated with - or adherent to solid particles, and attached to the rumen wall. Rumen content is thoroughly mixed via frequent rumen contractions to bring newly digested feed in contact with the bacteria in the rumen fluid. Free-suspended cellulolytic bacteria adhere to the fibrous substrate via the bacterial glycocalyx, adhesins or ligand formations [53] (Figure 1.4).

These pioneer species proliferate and encapsulate themselves in extracellular polymeric substances [118]. Cellulose, hemicellulose and other macropolymers fuel the metabolic activity in the biofilm and are converted into bacterial biomass and soluble intermediates, attracting secondary colonizers [111]. The solid adherent bacteria (primary colonizers) and bacteria loosely adherent/associated with the solids (secondary colonizers and biofilm members) are predominant and account for 80-90% of the total rumen microbial community [119,120] and the majority of digestive enzyme activities [121–124]. The solid-adherent environment is central in the rumen function and is characterized by a complex and divers bacterial community [125], responsible for the majority of the rumen fermentation. The free-living species in the liquid fraction contribute little to the metabolic activity and the rumen fluid serves as a relay of bacteria from the solid-adherent biofilms to newly ingested feed [126]. Beside the solid fraction and the rumen fluid, the epithelium is the third main environment in the ecosystem. Bacteria that attach to the epithelium are called ‘epimural’ bacteria. Because of their close proximity to the host, these species execute a variety of functions that have the potential to significantly modulate host health. They are involved in oxygen scavenging [127], hydrolysis of urea [128] and recycling of epithelial tissue [129].

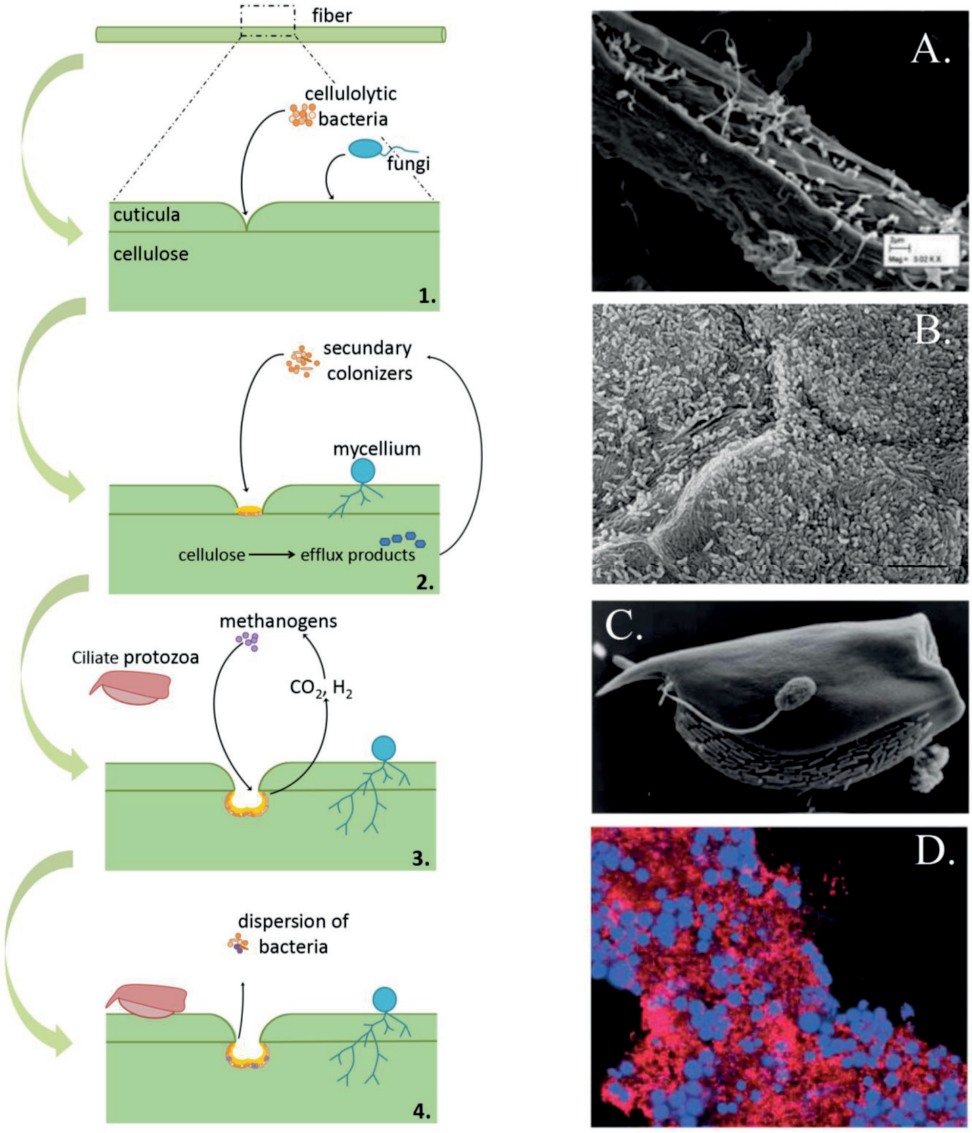


Figure 1.4 (**Left**) The chronological formation of a multispecies biofilm on freshly ingested fiber in the rumen ecosystem. **1.** Primary colonizers (cellulolytic bacteria) attach where the cuticle is damaged and the cellulose exposed. **2.** Metabolic end products from cellulose digestion attracts secondary colonizers to the growing biofilm. **3.** Methanogens appear as ball-shaped colonies within the biofilm. **4.** Bacteria and methanogens disperse from the mature biofilm. Ciliate protozoa and anaerobic fungi also attach to plant material and play an important role in fiber degradation. (**Right**) Microscopic images **A.** Bacteria attacking a plant fiber (SEM; L. Loubert, USDA publ.) **B.** Epimural bacteria adherent to the rumen epithelium (SEM; [130]) **C.** Ciliate protozoa with attached microbes and a fungal spore (SEM; M. Yokoyama & M.A. Cobos, USDA publ.) **D.** Bacteria (red) and Methanogens (blue) attached to crystalline cellulose (FISH-CLSM; [131]).

Similar to the bacteria, methanogens in the rumen can occur free-living in the rumen fluid, are found attached to the rumen epithelium or associated with the biofilm on particulate matter. Methanogens will be attracted to already established biofilms with locally elevated concentrations of H_2 and will occur as ball-shaped colonies on the maturing bacterial biofilm [131]. Beside these common environments, some methanogens enter into a symbiotic relationship and occur within (endosymbiosis) or attached to (episymbiosis) ciliate protozoa [132] in order to facilitate inter-species H_2 transfer. Methanogens that cohabit with ciliate protozoa were found to be responsible for 9-25% of the methanogenesis in the rumen fluid [133].

1.2.3 Factors influencing the rumen microbial communities

The composition and function of the microbial community is shaped by the dynamic physical, chemical, and predatory environment in the rumen of cows. The rumen microbial composition can be traced back to the first weeks of life. The developing rumen is initially inoculated with aerobic and facultative anaerobic bacteria, and are gradually replaced by exclusive anaerobic bacteria [134], methanogenic archaea, anaerobe fungi and ciliate protozoa [135]. Organisms are taken up from the environment, obtained from the feed or through contact with other animals. Only after several weeks, a microbial community is established that resembles the community in adult animals [134]. The mature rumen microbiome is prone to the influences of internal and external factors.

1.2.3.1 Influences of diet

The diet composition and the time intervals between feeding are the two main factors influencing the numbers and phylogenetic distribution of the microorganisms in the rumen. The dominating influence of nutrition is best illustrated by the consequences of a high-concentrate (i.e. cereal grain) diet, which has gained popularity in intensive agriculture to improve growth performance and increase production. Concentrate contains starch as main polysaccharide. The high availability of starch in the rumen supports the proliferation of amylolytic bacteria. Especially *S. bovis*, having a doubling time of around 20 min [27], will proliferate and produce massive amounts of lactate [63]. Lactate-utilizers cannot keep up with the increasing supply and lactate will accumulate causing the rumen pH to drop, a condition known as rumen acidosis [136]. A low pH (below 5.5) will inhibit the activity of bacteria sensitive to acidic environments, including cellulolytic bacteria and methanogens [137–139]. This example indicates that the diet exerts a profound influence on the microbial

community by providing specific substrates enriching those bacteria capable of efficiently consuming them, and by altering the rumen ambient conditions. The rumen pH is determined by the type of substrate (selecting for specific bacteria who produce specific VFAs), saliva production and removal of VFAs via absorption and passage from the rumen. Passage rate of particulates and saliva production is in turn influenced by the forage particle size. Larger fibers will increase chewing activity and rumination frequency and thus increase saliva secretion. Furthermore, larger particles are retained longer in the rumen (only particles less than 2 mm can pass to the omasum) thus increasing the passage rate. Although diet composition is the main determinant of the microbial community structure, a core rumen microbiome is present across a wide geographical range and similar bacteria and methanogens are observed in samples collected from different ruminant animals, receiving different diet types [103]. Also the Ruminomics project, in which rumen samples were collected from 1000 cows from 4 countries (UK, Sweden, Finland and Italy), concluded that nutrition, rather than the rumen microbiome, is the main driver of emissions [140]. Unsurprisingly, diet alterations and feed additives have been a popular choice to improve feed efficiency, increase production or lower methane emissions [141,142].

1.2.3.2 Breed specificity

Holstein-Friesian, Angus, Charolais, Shorthorn, Jersey, Belgian Blue, Blonde d'Aquitaine are some of the more than 800 cattle breeds recognized worldwide. Some breeds are specifically raised for beef while others are specialized in milk production and yet others have dual purpose. Cattle breeds will differ substantially in terms of morphological and physiological characteristics that evolved along with varying foraging behavior, climate and geographic diversity [143]. Considering that host adaptation plays an important role in regulating the rumen microbial community composition, it stands to reason that also the rumen microbial communities and fermentation characteristics are breed specific. For example, Jersey (JE) dairy cows have a higher reticulorumen weight as proportion of the body weight as compared to Holstein-Friesian (HF) dairy cows, which is correlated to a higher intake capacity and digestive efficiency [144]. Under similar dietary conditions, Paz et al. (2016) found that the majority (94.8%) of bacterial species are shared between lactating JE and HF cows indicating a common core of predominant bacteria. However, unique taxa were detected in both breeds suggesting a breed-specific subset of bacteria [76]. Rooke et al. (2014) measured H_2 and CH_4 emissions of two beef breeds under different dietary conditions. Aberdeen Angus-sired steers produced more CH_4 than Limousin-sired

steers, although this difference disappeared when emissions were expressed relative to DMI or gross energy intake, mainly because of the higher feed intake by Aberdeen-Angus. Both diet and genotype affected the abundances of several bacterial groups, quantified with qPCR assays [145]. Comparative studies between dairy or beef breeds suggest that the microbial community is adapted to the breed-specific rumen environment and the breed specific nutrition.

1.2.3.3 Host specificity

The effect of methane mitigation strategies is often subject to inter-animal variations [146,147] and providing identical feed to ruminants in the same herd does not necessarily result in identical methane emissions [116] nor are identical microbial communities established in the rumen [148–150], suggesting an influence of host related factors on the microbial community composition and activity in the rumen. These host related factors can be categorized into two general groups: (i) The genotype related factors, including those factors that could be influenced by gene expression or genetic heritability. This group comprises for example the size of the rumen organ, salivary production, absorption by the rumen epithelium and host-microbial cross-talk genes. (ii) Non-genotype related factors include the physiological state and the host's background (early life events as birth conditions, rearing strategy, weaning, previous diets and medical treatments). The combined effect of these genotype and non-genotype related factors can influence the passage rate, rumen pH and VFA concentrations and consequently also influence the microbial community profile. Weimer et al. (2010) was the first to study the host-specificity of the rumen bacterial community composition by cross-inoculating the rumen contents between cannulated cows. After a near-complete rumen content exchange between two cows, the bacterial community was followed using regular sampling and ARISA analysis (a PCR based fingerprinting technique). By this experiment, Weimer and colleagues found evidence of a host specific bacterial community composition [151]. Each cow is unique as it differs in morphological, physiological and behavioral characteristics. Adaptation to these host specific conditions plays a role in regulating the rumen microbial community composition.

1.3 IMPACT OF CROSS-CONTAMINATION OF ANIMAL FEED WITH ANTIMICROBIALS ON THE DEVELOPMENT OF RESISTANCE

1.3.1 Antibiotic use in pig industry

Advances in technology and sciences have opened the door for mass production of livestock, but the high animal densities of intensive livestock operations is conductive to the elevated prevalence of infections and the need for disease prevention strategies. In the 1950s, US scientists discovered that the administration of low concentrations of antibiotics as a feed additive promoted animal growth [152]. Many countries still permit the use of subtherapeutic administration of antibiotics as water or feed additives for growth promotion. This practice has been banned in the EU since 2006 [153] and recently the US followed with a new FDA veterinary Feed Directive [154]. However, the prophylactic and metaphylactic administration of therapeutic concentrations of antibiotics remain established in intensive agriculture. Unsurprisingly, a major part of the global antibiotic use occurs in agricultural settings. In 2001, the Union of Concerned Scientists estimated that, in the US alone, 24.6 million pounds (11.2 million kg) were used annually for non-therapeutic veterinary purposes compared to a mere 3 million pounds (1.4 million kg) for human medicine [155]. In Belgium, the total consumption of antibiotics in veterinary medicine has known a downward trend over the last decade (Figure 1.5) but still accounts for 242.4 tons in 2016, of which 36 tons was destined for antibacterial premixes. Over the years, over 99.6% of these antibacterial premixes were intended for medicated pig feed [156].

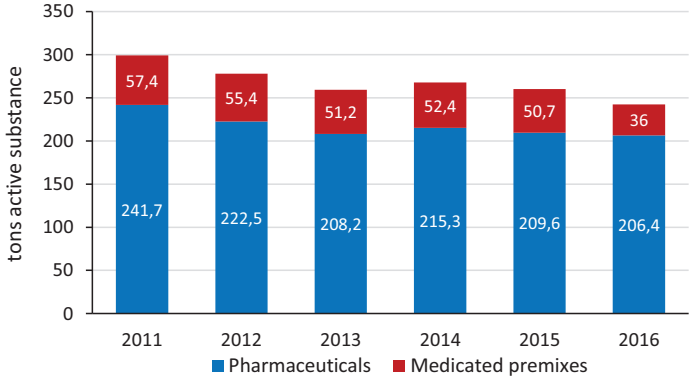


Figure 1.5 Total consumption of antibiotic compounds for veterinary use in Belgium for 2011-2016 [156]

Callens et al. (2012) collected data on antibiotic use from 50 Belgian pig herds in semi-closed production systems; 98% of the visited herds received at least one group level treatment, of which 93% prophylactic and 7% metaphylactic [157]. Group level treatments are administered to pig herds via oral treatment, administering medicated premixes as additive of feed or drinking water. The distribution of antimicrobials varies greatly and the choice of a specific antibiotic depends on the preferred administration route (oral or injectable), life stage (farrowing, battery, grower and finisher period), observed symptoms or prior disease outbreaks on the farm. The Belgian Veterinary Surveillance of Antibacterial Consumption (BelVet-SAC) reported that sulphonamides (sulfadiazine and trimethoprim) are the most frequently used antimicrobial class in veterinary premixes, followed by penicillins (amoxicillin) and tetracyclines (primarily doxycycline) (Figure 1.6) [156]. Many of the antibiotics frequently used in pig farming, are also listed by the WHO as critically important for human medicine.

Excessive antibiotic use is established in intensive livestock farming. By using, often preventive, group level treatments the farmer tries to reduce the risk of disease outbreaks and ensure a high production with limited costs. Unsurprisingly, livestock farming accounts for the lion's share of the global antibiotics use [158].

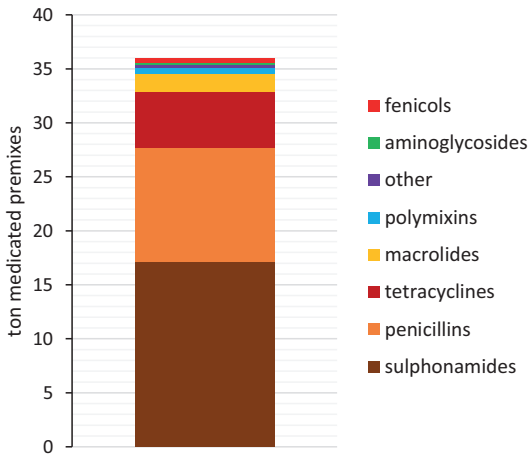


Figure 1.6 Total consumption of antibacterial premixes per class of antibiotics in 2016 in Belgium [156].

1.3.2 Cross-contamination

Despite governmental efforts to decrease subtherapeutic administration of antibiotics to livestock, cross-contamination of antimicrobial components to feed or water causes the exposure of non-target pigs to subtherapeutic antibiotics. The transfer of traces of an active antimicrobial substance contained in a medicated feed to a non-medicated feed, is referred to as "carry-over". The carry-over of an unintended substance to a feed is defined as "cross-contamination". Filippitzi et al. (2016) built a risk model to estimate the probability of cross-contamination. Assuming that medicated feed represents 2% of the total annual feed production, the model predicts that 5.5% of the produced feed would be cross-contaminated with various levels of antimicrobial compounds due to practices related to medicated feed; 29.7% of cross-contamination occurs during production (feed mill), 35.1% during transport and 35.2% happens on the farm [159]. Stolker et al. (2013) visited 21 feed mills in the Netherlands and collected and analyzed 140 samples of flushing batches, i.e. a feed mix produced directly after a medicated feed. Of these samples, 87% contained concentrations of antibiotics in the range of 0.1-154 mg/kg [160], which is the same range as the concentrations used for growth promotion (now banned in the EU and the US). From these results, the researchers estimated that 11% of the piglets, 38% of the pigs (< 50 kg) and 100% of lactating sows are exposed to cross-contaminated feed at least once a year [160]. The actual exposure will be much higher because Stolker et al. (2013) only took into account cross-contamination at the level of the feed mill, disregarding carry-over during transport or on the farm. In a follow-up study, Zuidema et al. (2014) collected 340 fecal samples (from 20 farms) at slaughter. Sixty days prior to slaughter, the animals did not receive medical treatments of any sort. However, 55% of the collected samples (and 80% of the farms) tested positive for at least one antibiotic. A broad range of antibiotics were detected with varying concentrations. Doxycycline was detected most often (103 samples), followed by oxytetracycline (49 samples), tylosin (50 samples) and sulfadiazine (33 samples) [161]. Banning administration of subtherapeutic doses of antibiotics as growth promoting agents has thus only solved half of the problem. Carry-over from medicated feed to non-medicated feed or water unintentionally exposes pigs to subtherapeutic concentrations of antibiotics. A covenant from 2013 between the Belgian Federal Agency for the Safety of the Food Chain (FAVV) and the association of Belgian compound feed manufacturers (BEMEFA) stipulates specific agreements to reduce occurrence and levels of cross-contamination to as low as reasonably achievable, without excessively increasing

production costs. The covenant aims to limit carry-over of antimicrobial compounds to a maximum limit of 1% of the therapeutic dose. To achieve this goal, as of January 2014, antimicrobial premixes are no longer added and mixed in the main mixer of the production line but rather in an end-of-line mixer or a mobile mixer (fine-dosing system) on the transport truck. Also compliance to Good Manufacturing Practices, prudence when processing medicated feed and improved production and transport systems can further minimize carry-over.

1.3.3 Antimicrobial resistance

1.3.3.1 Acquisition and transfer of resistance

Antimicrobial resistance (AMR) is the ability of a microorganism to withstand or stop the effects of an antibiotic compound. A population of bacteria within the same species will have a certain genetic diversity. While most of the wild type bacteria will be susceptible to an antibiotic, some individuals have the ability to resist its effects, because of a random mutation in a target gene or by the presence of mobile genetic elements containing genes encoding antibiotic resistance. Administration of an antibiotic at a concentration above the minimum inhibitory concentration (MIC) will exterminate most of the susceptible (wild type) bacteria, creating a vacuum for the few insensitive and resistant bacteria to proliferate and replace the wild type species. The various strategies to resist an antibiotic compound are all genetically encoded and two main categories are distinguished: (i) Intrinsic resistance is the innate ability of a bacterial species to withstand the effect of an antibiotic compound through its inherent structural or functional characteristics. A species can be insensitive to an antibiotic (i.1) when it lacks the target or uptake mechanisms for the antibiotic, for example: aminoglycosides are mostly inactive against anaerobic bacteria because they lack the oxidative metabolism necessary to support sufficient uptake of the compound [162]; (i.2) when the cell is inaccessible for the antibiotic, for example vancomycin cannot penetrate the outer membrane of Gram-negative bacteria, making them intrinsically resistant [163]; (i.3) when the species has chromosomally encoded resistance genes. (ii) Acquired resistance includes (ii.1) random changes in the bacterial genome through a mutation in a gene that encodes an antibiotic target or far more frequently, (ii.2) the acquisition of mobile genetic elements via horizontal gene transfer. Horizontal gene transfer is defined as the movement of genetic material from one microorganism to another, which occurs through three well-understood mechanisms: transformation, transduction and

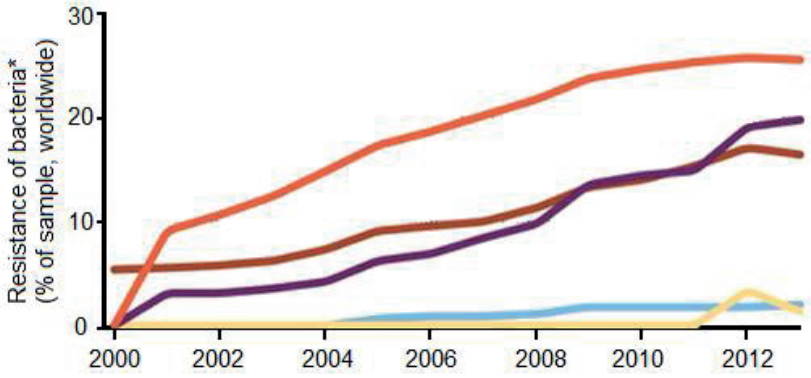
conjugation. Transformation is the uptake of free-suspended exogenous DNA from the environment by bacteria and archaea. Transduction is the delivery of genetic material via phage predation [164]. Conjugation is considered to be the most important way of AMR transfer and involves direct contact between the donor and the recipient cell via a bridge-like connection (F-pilus) and is therefore subject to a few restrictions:

- Conjugation involves only the transfer of specific mobile genetic elements, most often a plasmid or transposon, carrying approximately 40 genes involved in F-pilus synthesis on the surface of the donor and connection with the surface of a compatible recipient [165]. The F-plasmid can replicate autonomously using its own origin of replication (*ori*) or integrate itself into the bacterial chromosome by homologous recombination [166]. Unlike plasmids, transposons cannot exist independently but can "jump" from plasmid to plasmid, from plasmid to the chromosome (or vice versa) or within the chromosome by replicative or 'cut-and-paste' mechanisms mediated by the enzymatic activity of transposases [167].
- The origin of replication on a plasmid (ORI) regulates the copy numbers of this specific plasmid, but also imposes a restriction on the number of different plasmids because plasmids encoding the same ORI are incompatible with one another and cannot coexist in the same cell [168]. Plasmids are therefore classified into incompatibility groups (Inc) based on their replication and partitioning systems.
- Conjugative transfer requires spatial proximity of the donor and recipient.

With horizontal gene transfer, prokaryotes can transfer genetic material and expand their genetic capacities. Especially plasmids function as a platform where gene arrays are assembled, reasserted and spread to neighboring bacteria through conjugation. The accretion of useful plasmid-encoded genes can permit a bacterial strain to survive a toxic environment (R-plasmids) [169], produce bacteriocins to kill competing bacteria and safeguard a niche for themselves [170], enable metabolism of rare substances [171] or provide virulence [172]. A particularly striking example is plasmid encoded antibiotic resistance, which is responsible for the rapid emergence of multiple drug resistant bacteria, enhanced by selective pressure from human and veterinary antibiotic use.

1.3.3.2 Resistance development in the GIT of pigs

Orally administered antibiotics via medicated feed/water or cross-contaminated feed, will pass the intestinal tract before reaching systemic circulation. Concentrations in the gut compartments depend on the initial concentrations in the feed, as well as the pharmacokinetic properties and bioavailability of the drug. The bioavailability (F) is the fraction of an administered dose that is absorbed and enters the blood stream (intravenous injection thus gives a 100% bioavailability). A lower bioavailability of a drug relates to higher concentrations in the intestinal tract and in the feces. Peeters et al. (2016) compared the oral bioavailability of three commonly used veterinary antibiotics. Chlortetracycline had the lowest F (6%) and thus the highest transfer ratio to the feces, doxycycline had a F of 21-50% and sulfadiazine had the highest F of 85-100% [173]. It is estimated that 75-90% of antibiotics used in livestock production are excreted from animals, mostly unmetabolized, and enter the environment, the sewage systems and water sources [174]. Prolonged exposure of antibiotics creates selective pressure for the propagation of resistant species/strains within the gut microbial community. Looft et al. (2011) raised pigs in a controlled environment with one experimental group receiving a diet containing performance-enhancing antibiotics ASP250 (chlortetracycline, sulfamethazine and penicillin) and a control group receiving the same diet without additives. During antibiotic treatment, the microbial community shifted to higher abundances of *Proteobacteria*, driven by an increase of *Escherichia* and *Shigella*. QPCR analysis further indicated that AMR genes increased in abundance and diversity in the microbiome of the medicated pigs [175]. Some of the enriched AMR genes confer resistance to antibiotics that were not administered, suggesting that the selected resistance genes (those conferring resistance for the administered antibiotics) were on plasmids that also contained resistance genes against other antibiotics, a phenomenon called “co-selection”. Also the administration of subtherapeutic concentrations of tylosin (class of macrolides) causes taxonomic shifts in the gut microbial communities of pigs [176,177]. However, not all antibiotics induce changes to the microbial community composition. For example, subtherapeutic administration of chlortetracycline resulted in only minor taxonomic shifts and a gut microbial community composition that was not significantly different from untreated pigs [177].



**Enterobacteriaceae*, incl. *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter* and *Salmonella*

Figure 1.7 Graphical representation of the increasing proportion of indicator species that display resistance to common antibiotics: Fluoroquinolones, Cephalosporins (3rd gen.), Aminoglycosides, Carbapenems and Polymyxins (incl. colistin), based on resistance rates for isolates from blood and cerebrospinal fluid form patients worldwide [178].

The prevalence and degree of antibiotic resistance in the intestinal microbiomes of pigs is often measured by quantifying specific AMR genes in fecal samples or by cultivation of an indicator species on selective media containing an antibiotic. Van den Bogaard et al. (2000) collected 1321 fecal samples from pigs at Swedish and Dutch abattoirs and quantified resistant *E. coli* and enterococci on selective agar without or with one of nine tested antibiotics. In the Dutch samples, the cultivated *E. coli* species showed high prevalence of resistance against amoxicillin (70-94% of the isolates displayed resistance against amoxicillin), oxytetracycline (78-98%), trimethoprim (62-96%), chloramphenicol (55-67%) and neomycin (38-67%). The percentage Swedish samples with high degree of resistant *E. coli* was significant lower, reflecting the differences in antibiotic use between both countries [179]. In 2015, also plasmid-encoded colistin resistance was reported for the first time during a routine surveillance project on AMR in commensal *E. coli* from livestock animals in China [180]. The prevalence of the colistin resistance gene mcr-1 has since then been reported in several other countries [ex.: 181,182,183,184,185]. Mutations that confer resistance to colistin had been reported previously and many bacteria occurring in agricultural settings are known to be resistant to colistin, but the existence of transferable colistin resistance by plasmid-mediated *mcr-1*, *mcr-2* and *mcr-3* [186] is problematic as colistin is considered as one of the "last-resort" drugs against multiple drug resistant

pathogens in human medicine. Undeniably, coinciding with intensive livestock production and its antibiotic use is the continuously increasing prevalence of bacterial resistance against those antibiotics (Figure 1.7).

Antibiotic resistance is not a problem that remains within the borders of the farm. Resistant bacteria can spread beyond the agricultural settings and pose a major threat to public health. Three pathways are distinguished by which resistant bacteria can spread widely and rapidly from farm animals to humans:

i. Farmers or animal caretakers, slaughterhouse workers and veterinarians are at risk of being colonized with resistant bacteria via close contact with animals and their excrement. They provide a conduit for the entry of AMR genes into the broader community [174]. This type of transmission was first reported by Levy et al. (1976) who found the same tetracycline-resistant *E. coli* strains in the intestinal communities of farmers as in the chickens fed tetracycline-supplemented feed [187]. Aubry-damon et al. (2004) assessed the quantitative contribution of pig farming to antibiotic resistance in the commensal communities of farmers by comparing 113 healthy farmers to 113 non-farmers. Pharyngeal carriage of macrolide resistant *Staphylococcus aureus* and penicillin resistant streptococci were significantly more detected in farmers. Intestinal isolation of *Enterobacteria* resistant to nalidixic acid, chloramphenicol, tetracycline, and streptomycin was also higher in farmers compared to non-farmers, underlining the significant association between livestock farming and exposure to resistant bacteria [188].

ii. Consumers may be exposed to resistant bacteria via contact or consumption of animal products, a far more complex route of transmission. Although proper cooking and hygiene may eliminate most of the contaminating bacteria, undercooked or raw meat may serve as a vector for AMR bacteria to humans. The rise of drug-resistant bacteria in final meat products has been well-documented [189–191] and has been correlated to the increased incidence of infections with drug-resistant foodborne pathogens, such as *Salmonella* [192–194].

iii. Consumption of vegetables, especially when eaten raw, represents a route of direct exposure to bacteria in the soil. Crops can become contaminated with drug resistant bacteria through soil fertilization with manure [195] or the use of contaminated water. Even long after fertilization, the soil can maintain high numbers of resistant bacteria and resistance genes. Environments polluted with manure form intensive livestock are a reservoir of AMR genes and even in the absence of antibiotics, these AMR genes remain persistent in bacterial populations [196,197].

1.3.4 Doxycycline – a popular antibiotic in pig husbandry

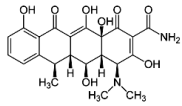
1.3.4.1 Doxycycline in pig husbandry

Four tetracycline antibiotics are commonly used in veterinary medicine: tetracycline, chlortetracycline, oxytetracycline and doxycycline. Tetracyclines, mainly represented by doxycycline, accounted for 32.8 % of the sales of veterinary antibiotics in 30 EU and EEA countries in 2015, in 90.8% of the case as feed or water additive (premix, oral power or oral solution) [198]. However, when considering these kinds of data it is important to realize that dosing of various antibiotic agents between and within classes, vary substantially. For example, the dosage of doxycycline is about a quarter of a dose of oxytetracycline [198], meaning that more animals can be treated with equal quantities of doxycycline. Doxycycline is a popular choice in livestock production and often used to treat or prevent respiratory infections.

1.3.4.2 Characteristics of doxycycline

Doxycycline (Vibramycin®) was discovered and developed by Pfizer and received FDA-approval for clinical use in 1967. Doxycycline (DOX) is synthetically derived from oxytetracycline (tetracycline class) and was found to have clinical effectiveness against infections caused by susceptible strains of Gram-positive and Gram-negative bacteria and certain protozoa [199]. Doxycycline, commonly administered as a hyclate salt, is 5-10 times more lipophilic than other tetracyclines resulting in higher tissue penetration and better antimicrobial properties due to improved entrance into bacterial cells [200]. After oral administration, doxycycline hyclate is rapidly absorbed from the gastrointestinal tract and is widely distributed in the body. Doxycycline hyclate is one of the most commonly used (tetracycline) antibiotics in pig rearing because of its high bioavailability (Table 1.3).

Table 1.3 Characteristics of doxycycline hyclate salt

Doxycycline hyclate	
Occurrence	Light-yellow crystalline powder
Molecular formula	$(C_{22}H_{24}N_2O_8)_2 \cdot C_2H_6O \cdot H_2O$
Molecular weight	1025.89 M
Oral bioavailability (F)	10-30 % [173,201,202]
Therapeutic dose (pigs)	10.5 mg kg^{-1} BW day^{-1}
Structure (DOX)	

1.3.4.3 Mechanism of action

Similar to other tetracyclines, the broad spectrum antimicrobial effects of DOX are based on the inhibition of prokaryotic protein synthesis. After entering the cytoplasm, DOX binds to the 30S subunit of the ribosomes and interferes with the attachment of aminoacyl-tRNA to the mRNA-ribosome complex, thus preventing translation. The association of DOX (and tetracyclines in general) with the ribosome is reversible and the effect of tetracycline is thus merely bacteriostatic. To interact with their target (i.e. the ribosome), DOX must pass one or more membrane barriers, depending on whether the susceptible bacteria is Gram-positive or Gram-negative. Tetracycline traverses the outer membrane of Gram-negative bacteria through OmpF and OmpC porin channels as positively charged cation-tetracycline complexes. In the periplasm, the tetracycline complex dissociates into uncharged tetracyclines, which due to its lipophilic nature (especially doxycycline) can diffuse through the inner membrane. Similarly, the uncharged lipophilic molecule is likely to transfer across the single cytoplasmic membrane of Gram-positive bacteria [203].

1.3.4.4 Mechanisms of resistance

Antibiotics have existed probably as long as there have been bacteria. Mass spectroscopic identification of tetracycline in the skeletal remains of the ancient population of Sudanese Nubia (350-550 CE), suggested that Nubians drank beer laced with antibiotics [204]. In nature, tetracyclines are produced by certain strains within the genus of *Streptomyces* [205], a slow growing bacteria that is predominantly found in soil. *Streptomyces* is renowned for its wide range production of bioactive secondary metabolites and is responsible for the majority of the clinically useful antibiotics. The production of antibiotics gives the slow-growing *Streptomyces* a selective advantage in the competition with other bacteria in the ecosystem [206]. But, while some bacteria developed the capability to produce antibiotics, others developed mechanisms to protect them against the negative effects of these antibiotics [207].

Point mutations in the chromosomal genes, resulting in an altered 16S rRNA structure [208,209] or membrane permeability [210], can render a species insensitive to tetracyclines. However, tetracycline resistance is more often due to the acquisition of new genes associated with mobile plasmids or transposons. There are two main mechanisms of acquired tetracycline resistance: energy-dependent efflux pumps and ribosomal protection

proteins. A third mechanism, involving enzymatic inactivation/degradation of tetracycline, has been described although the clinical relevance of this type of resistance is unclear [211].

Efflux pumps are transmembrane transporter proteins involved in the active extrusion of toxic compounds out of the cytoplasm and back into the extracellular environment. These proteins are found in both Gram-negative as Gram-positive bacteria and they are encoded by a diverse range of genes (Table 1.4), suggesting also a range in pump characteristics. Five major families of efflux pumps are known: major facilitator superfamily (MFS); multidrug and toxic efflux (MATE); resistance-nodulation-division; small multidrug resistance (SMR) and ATP binding cassette (ABC) [212,213]. Tetracycline efflux proteins belong to the major facilitator superfamily [214] and catalyze the extrusion of cytoplasmic tetracycline-divalent metal complexes coupled to proton translocation (antiporter type efflux) [215,216].

Ribosomal protection is mediated by soluble cytoplasmic proteins that bind the ribosome, causing an alteration in ribosomal conformation that prevents tetracycline from binding. *Tet*(O) and *tet*(M) can even dislodge tetracycline bound to the ribosome. Tetracycline antibiotics are either released from the ribosome or prevented from attaching, thus safeguarding the translation activity [217]. A total of 46 different acquired tetracycline resistance genes have been identified in 126 genera [218]. Phylogenetic analysis of the *tet* genes encoding ribosomal protection proteins and transmembrane efflux revealed the monophyletic origin of these genes, with each phylogenetic branching event separating one class of *tet* genes from another [219,220]. Most of the *tet* genes are associated with mobile plasmids and transposons, which also encode their own transfer, which presumably influences the range of acceptors to mostly taxonomically related species. The evolutionary history of *tet* genes and the limitations of transfer cause most *tet* genes to have a host preference and will be found in some taxonomic lineages while absent in others (Table 1.5). *Tet*(M) has the broadest taxonomic distribution and has been detected in both Gram-negative and Gram-positive bacteria, refuting the hypothesis of a physiological barrier for exchange between Gram-positive and Gram-negative bacteria [221,222]. Similarly *tet* genes that were previously labelled as Gram-positive associated (such as *tet*(K)(L)(O)), are increasingly detected in Gram-negative bacteria, and vice versa (*tet*(Q)). In general, bacteria that are resistant to doxycycline are also resistant to other types of tetracyclines [223].

Table 1.4 Mechanisms of resistance against tetracycline antibiotics of *tet* and *otr* genes, adopted from https://faculty.washington.edu/marilynr/

Efflux (33)	**Ribosomal protection (12)**	**Enzymatic inactivation (13)**	**unknown**
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(59)	*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32)	*tet*(X)	*tet*(U)
tet(G), tet(H), *tet*(J), *tet*(V), *tet*(Y)	*tet*(Q), *tet*(T), *tet*(36)	*tet*(37)	
tet(Z), *tet*(30), *tet*(31), *tet*(33), *tet*(57)	*otr*(A), *tet*B(P)	*tet*(34)	
tet(35)	*tet*(44)	*tet*(47), *tet*(48), *tet*(49), *tet*(50)	
tet(39), *tet*(41)		*tet*(51), *tet*(52), *tet*(53), *tet*(54)	
tet(K), *tet*(L), *tet*(38), *tet*(45), *tet*(58)		*tet*(55), *tet*(56)	
*tet*A(P), *tet*(40)			
otr(B), *otr*(C)			
tcr(3)			
tet(42)			
tet(43)			
*tet*AB(46)			
tet(AB(60)			

1.3.4.5 Persistence of tetracycline resistance

The inverse correlation between plasmid copy numbers and growth rate suggest that plasmid maintenance imposes a metabolic burden for the host species [224]. Unless the plasmid encodes genes that provide a selective advantage, bacteria without plasmids will outgrow those bacteria that need to invest resources to maintain a plasmid. Therefore, in absence of a selective pressure of an antibiotic, antibiotic resistance genes are expected to disappear from the ecosystem as the population selects for those bacteria lacking the plasmid. Nevertheless, Tamminen et al. (2011) found that the prevalence of certain *tet* genes remained elevated several years after antibiotic use ceased [197]. The persistence of acquired resistance in the absence of selective pressure could be attributed by a number of factors. Rysz et al. (2013) investigated tetracycline resistance gene maintenance under varying conditions of continuous culture. *Pseudomonas aeruginosa* populations completely lost their plasmids (*tet* carrying RT1; 56 kb) in absence of tetracycline. Under limited nutrition and anaerobic conditions, the loss of plasmid encoded *tet* genes occurred at a much higher rate. In contrast, *E. coli* maintained the presence of a much smaller plasmid (*tet* carrying pSC101; 9.3 kb) even after 500 generations without tetracycline, although at much lower levels. The persistence of plasmid encoded *tet* genes is thus positively influenced by better growth conditions such as an aerobic environment with sufficient nutrients and the size of the plasmids as smaller plasmids impose a smaller burden. Furthermore, residual concentrations of antibiotics and other chemical stressors for which the plasmid may provide resistance, can also contribute to the maintenance of the plasmid (i.e. co-selection) [225]. Careful regulation of expression and partitioning and/or addiction systems might also contribute to long-term prevalence of *tet* genes [226].

Table 1.5 Distribution of *tet* genes among bacterial genera identified in fecal samples of pigs using amplicon sequencing. (based on https://faculty.washington.edu/marilynr/)

Bacteroidetes (Gram-negative)			
Wautersiella	*tet*(X)	*Bacteroides*	*tet*(M)(Q)(W)(X)(36)
Prevotella	*tet*(M)(Q)(W)		
Firmicutes (mostly Gram-positive, except *Megasphaera* and *Selenomonas*)			
Anaerococcus	*tet*(M)	*Clostridium*	*tet*(K)(L)(M)(O)(P)(Q)(W)(36)(40)(44)
Catenibacterium	*tet*(M)	*Enterococcus*	*tet*(K)(L)(M)(O)(S)(T)(U)(58)
Erysipelothrix	*tet*(M)	*Lactobacillus*	*tet*(K)(L)(M)(O)(Q)(S)(W)(Z)(36)
Aerococcus	*tet*(M)(O)	*Peptostreptococcus*	*tet*(K)(L)(M)(O)(Q
Roseburia	*tet*(W)	*Streptococcus*	*tet*(K)(L)(M)(O)(Q)(S)(T)(U)(W)(32)(40)AB(46)
Ruminococcus	*tet*(Q)	*Anaerovibrio*	*tet*(O)(Q)
Acidaminococcus	*tet*(W)	*Megasphaera*	*tet*(O)(W)
Butyrivibrio	*tet*(O)(W)	*Mitsuokella*	*tet*(Q)(W)
Selenomonas	*tet*(M)(Q)(W)	*Veillonella*	*tet*(A)(L)(M)(Q)(S)(W)
Proteobacteria (Gram-negative)			
Actinobacillus	*tet*(B)(H)(L)(O)	*Acinetobacter*	*tet*(A)(B)(G)(H)(L)(M)(O)(W)(Y)(39)
Campylobacter	*tet*(O)(44)	*Escherichia*	*tet*(A)(B)(C)(D)(E)(G)(J)(L)(M)(W)(Y)(X)
Vibrio	*tet*(A)(B)(C)(D)(E)(G)(K)(M)(34)(35)		
Actinobacteria (Gram-positive)			
Bifidobacterium	*tet*(L)(M)(O)(W)	*Microbacterium*	*tet*(M)(O)(42)
Tenericutes (Gram-negative)			
Mycoplasma	*tet*(M)		
Spirochaetes (Gram-negative)			
Treponema	*tet*(B)		
Fusobacteria (Gram-negative)			
Fusobacterium	*tet*(G)(L)(M)(O)(Q)(W)		

1.4 TECHNIQUES TO STUDY THE MICROBIAL ECOSYSTEM OF THE GASTROINTESTINAL TRACT

1.4.1 Sample collection

1.4.1.1 Rumen sample collection

Imperative in the study of the rumen microbial ecosystem is sample collection. Sampling the rumen contents can be done in several ways: (i) By using an oral stomach probe. The probe is passed through the mouth, further down the esophagus and into the rumen. However, this method of sampling is prone to some drawbacks. Depending on the insert depth, rumen fluid will be collected from the cranial dorsal (180 cm) or the central rumen (200 cm) [227]. The sampled rumen fluid might not always be representative or reproducible because the sample collector does not have a clear view of where the sample is collected and different rumen fractions have varying fermentation parameters [227], also samples can become "contaminated" with saliva. Furthermore, an oral stomach tube is not capable of collecting samples from the fibrous material or the epithelium. Alternatively, (ii) cannulated cows offer a range of benefits. When a cow has been surgically fitted with a cannula, a rubber-sealed porthole provides direct access to the rumen, allowing collection of rumen fluid using a tube or perforated probe in combination with a vacuum pump or suction device. By replacing the sampling probe, a representative rumen fluid sample can be collected with a clear view of the location. Beside fluid collection, samples can be obtained from the fibrous material and the epithelium as well. However, where oral stomach probing can be used for all animals, fitting a cannula requires an invasive surgical procedure and is therefore a costly investment and only applicable to adult animals. As a third alternative, (iii) Tapio et al. (2016) proposed oral sample collection of regurgitated digesta (bolus) as non-invasive proxy for assessing the rumen microbial community [228], although to our knowledge this manner of sample collection has not yet been used after the initial publication. A fourth option, although not commonly used, (iv) is the sample collection after slaughter. Sampling is limited to one sampling time and therefore not suited for longitudinal studies but provides easy excess to all rumen environments, allows easy homogenization of the rumen contents and additional data can be collected simultaneously (ex.: rumen quantity, rumen size, size of epithelial papillae). In addition to *in vivo* experimentation, the rumen microbial ecosystem can be mimicked in an *in vitro* chemostat.

The RUSITEC system allows long-term and stable *in vitro* fermentation under similar nutritional and ambient conditions as in the rumen [229] and has been a popular alternative to study the influences of variable conditions (ex. temperature, pH), feed additives, etc. on the microbial digestion and methane production [ex. 228,229].

1.4.1.2 Sample collection of the pig GIT

Sample collection from the pig's gastrointestinal tract can also be done via cannulation of the pig [232], though this occurs much less frequently than with cattle. Therefore, collection of intestinal content is usually limited by endpoint sampling at slaughter, which does not allow longitudinal studies. Instead, scientists turn to fecal samples as a proxy for the gut microbial ecosystem. Furthermore fecal analysis can provide additional insight into one of the main routes of the spread of resistant bacteria to the environment. Alternatively, the effects of antibiotics on the microbial ecosystem of the gastrointestinal tract can be investigated using *in vitro* simulations of intestinal compartments. By means of reactor setups, feed medium and appropriate inoculation material, the physical and biochemical parameters of the pig's intestinal microbial ecosystem, or part of it, can be accurately simulated. These *in vitro* models ease sample collection and increase repeatability of experiments under standardized conditions.

1.4.2 Methods to study the microbial community

1.4.2.1 Cultivation techniques

Much of our knowledge about intestinal microbial ecosystems is derived from culture-based experiments. In these studies, specific species/strains are isolated from the complex intestinal microbiome and their metabolic capacities, growth conditions and dependencies are investigated in a strictly controlled and artificial environment. Combining two bacterial strains in batch or continuous culture experiments allow investigating interactions like synergy or competition. Isolation and cultivation of single bacteria has it merits for many purposes like screening for the presence of pathogens and identifying and characterizing novel species. However, using plate cultivation to study a complex microbial ecosystem easily overlooks the true complexity of the microbial ecosystem: fluctuating conditions, interaction between multiple species and predation. Furthermore, studies based on plate cultivation are often limited by the cultivability of bacteria, a phenomenon known as the "great plate count anomaly", corresponding to the difference between microscopic and

cultivation counts. Effectively, only a small fraction of the diverse microbial community can be isolated and grown on artificial media. Nevertheless, cultivation remains a popular technique in the study of antibiotic resistance as they are able to enumerate specifically those bacteria possessing resistance against a specific antibiotic. Often these studies focus on indicator species such as *E. coli* to monitor resistance development.

1.4.2.2 Molecular techniques

Studies implementing culture-independent microbial profiling are not limited by cultivability and can investigate the bacterial community as a whole. Molecular microbiology methods use DNA to identify and quantify bacterial species or taxonomic groups and have known an explosive development in the last few decades.

1.4.2.3 Quantitative analysis.

Real-time (quantitative) PCR can be used to quantify the numbers of a specific gene by real-time monitoring the amplification of the targeted DNA molecule during PCR. Two common detection methods are used for qPCR: non-specific fluorescent dyes that intercalate in the double-stranded amplicons (i.e. SYBR) or fluorogenic probes specific for a target sequence. Intercalating dyes are often the choice for quantifying larger taxonomic groups like total bacteria or total methanogens.

1.4.2.4 Qualitative analysis.

Community profiling techniques use the sequences of conserved genes to identify taxonomic groups and differentiate between species. Commonly, the 16S rRNA gene is used for phylogenetic studies. 16S rRNA is a necessary constituent of the 30S small subunit of prokaryotic ribosomes and thus omnipresent in all bacteria and archaea (mostly with multiple copies per chromosome) and contains conserved and hypervariable regions. While 16S rRNA gene sequencing is a useful approach to take a census of the taxonomic composition and the richness and diversity, it provides no functional information other than that which is ascribed to the identified taxa (usually by cultivation studies or genomic analysis of representative strains). To a great extent, sequencing is still dependent on species isolation to produce a reference set of microbial sequences and to study the function of genes. The growing number of sequencing-based studies has highlighted the abundance of uncultured and thus unknown taxa.

PCR-based fingerprinting techniques, such as DDGE and ARISA, were the first available methods to investigate the microbial diversity and community composition. Profiling the microbial community of a complex ecosystem like the intestinal tract of pigs or the rumen of cows is restrained by the many shortcomings of these techniques. These community-fingerprinting techniques give a representative overview of the species present without providing taxonomic information. Furthermore, the true richness and diversity is often underestimated as only the dominant members of the community are observed in the profiles. These techniques have fallen out of favor with the upcoming of next generation sequencing (NGS). Although NGS techniques still share some of the limitations of community fingerprinting techniques as they are also dependent on DNA extraction and PCR, they can identify the species present in the community and provide higher resolution and sensitivity [233].

Amplicon sequencing is a highly targeted approach for analyzing genetic variation in the bacterial 16S rRNA gene across multiple species. The GS20 Sequencer, released by 454 in 2005, marks the beginning of amplicon sequencing in phylogeny and taxonomic studies. In recent years, Illumina sequencing technology has surpassed Roche's 454 in performance and cost efficiency causing Roche to discontinue the support of its 454 sequencing platform in 2016. Nowadays, Illumina's MiSeq sequencing platform is the most widely used for metabarcoding although other sequencing techniques such as Ion Torrent (Life Technologies) and SOLiD (Life Technologies) are commercially available.

1.4.2.5 Data processing methods for metabarcoding

Taxonomic composition. The taxonomic composition is commonly visualized by means of a bar chart of the different taxonomic groups (at a certain taxonomic level: Phylum, Order, Class, Family, Genera) or summarized in tables.

Alpha diversity. The α-diversity refers to the species richness (number of taxa) and diversity (number of taxa and their relative abundance) within a single ecosystem, i.e. a sample collected from a specific environment at a specific time. Frequently used indices are the OTU count (number of OTUs with relative abundance > 0%) within a sample to indicate the richness. Alternatively, richness estimation methods can also correct for an insufficient sequencing depth, that is unable to detect low-abundant species. These estimators, such as the Chao1 index, ACE and ACE1, use the number of OTUs that are only present with one or two reads to calculate the number of OTUs that presumably

remained undetected [234]. Rarefaction analysis is a third technique to assess species richness in function of the sequencing depth by the construction of rarefaction curves. A plateaued curve indicates the sequencing depth (i.e. number of sequenced reads) was sufficient to observe the entire community. The diversity takes into account the species richness as well as the evenness. The Shannon index is a statistical index that assumes all detected species are randomly sampled and is calculated as $H = -\sum_{i=1}^{n} p_i \ln(p_i)$, with p_i representing the proportion of each individual species and n the number of different species. The Simspon index on the other hand, gives more weight to dominant species and is calculated as $D = \frac{1}{\sum_{i=1}^{n} p_i}$.

Beta diversity. The β-diversity was originally defined as the extent of change in community composition or degree of community differentiation, in relation to a complex-gradient of environment or a pattern of environments [235]. The community differences between samples can be visualized by a clustered heatmap, which also gives an idea of the richness and relative abundances of individual taxa. Ordination is also a frequently used technique to visualize compositional differences of communities from different ecosystems, treatments or time points. UniFrac is an effective distance metric for microbial community comparisons and uses phylogenetic information to quantify community similarities. UniFrac distances coupled with standard multivariate statistical techniques such as principle coordinates analysis (PCoA) spread samples in a multidimensional space (usually 2 or 3D) based on community similarities [236]. Similarly, the Bray-Curtis dissimilarity matrix can be used to quantify the compositional differences between the communities of two samples based on species count (i.e. OTUs) and their relative abundances in each community, and gives pairwise community dissimilarities as percentages. Non-metric multidimensional scaling in combination with Bray-Curtis is a popular ordination approach for graphically representing relationships between samples in a multidimensional space. Many alternatives exist for ordination (RDA, CA, DCA, CCA, etc.) and for distance/(dis)similarity index (Jaccard, Eucidean, Manhattan, Canberra, etc.).

Microbial co-occurrence relationships. The intestinal microbial ecosystem comprises a very diverse microbial community represented by many bacteria with often competitive or cooperative interactions. Programs exist to detect significant non-random patterns of co-occurrence (copresence and mutual exclusion) in incidence and abundance data of bacteria,

in order to explore interactions between organisms and environmental effects on coexistence [237].

1.4.3 Parameters of fermentation activity

1.4.3.1 Volatile fatty acid analysis

Short chain carboxylic acids are important intermediates and metabolites of anaerobic fermentation. Carboxylic acids with 2 to 7 carbon atoms are referred to as volatile fatty acids (VFA). The presence and concentration of VFAs in an environment is a proxy for the fermentative activity of the bacteria, which is influenced by ambient conditions and diet type. The principle VFAs in the rumen and the large intestine are acetate, propionate and butyrate in ratio's ranging from 75:15:10 to 40:40:20 [238]. These short chain fatty acids are mainly produced by the fermentation of dietary carbohydrates. Proteolysis generates a complex mixture of metabolic end-products, including the main VFAs (acetate, propionate and butyrate) and branched chain fatty acids such as isobutyrate and isovalerate [239,240].

Volatile fatty acids can be identified and quantified with high accuracy and sensitivity using chromatography.

1.4.3.2 Methane production

Enteric methane production from cattle livestock is a major contributor to greenhouse gas emissions. Unsurprisingly, methane mitigation has been the topic of many studies. Accurate methane measurement of individual cows is a necessity and at the ILVO Animal Sciences Unit, two measurement methods are available:

i. Open-circuit chambers (Figure 1.8) provide an ideal platform for measuring methane emissions from individual cows. Cows are housed in individual chambers for a consecutive time (usually 3 to 4 days) during which CH_4, CO_2, N_2O and NH_3 is measured at regular intervals in the exhaust gas from each chamber. A ventilation system generates an airflow in the chamber and forwards the exhaust gas to an absorption spectrometer for analysis. Although measurements in open-circuit chambers only collect data over a limited time and under specific circumstances, the continuous measurement provides accurate data on the daily methane emissions as it takes into account the diurnal fluctuations of methane emissions. At ILVO, six cows can be measured simultaneously [241].

ii. Alternatively, the GreenFeed system (C-Lock, Figure 1.8) measures methane in the breath of cows. The GreenFeed provides fixed portions of concentrate to individual cows at regular intervals. The methane is measured while the cow is eating the concentrate. This system therefore only measures during several short periods spread over a day. On the upside, the GreenFeed allows prolonged measurements in a loose-housing facility of a larger herd, roughly 30 to 35 cows.

Figure 1.8 (**Left**) Photo of a dairy cow in an open-circuit respiration chamber at ILVO. (**Right**) Photo of a dairy cow eating concentrate in the GreenFeed while methane is measured in his breath

iii. Another commonly used system for methane measurements is a variant of the GreenFeed principle, where methane is measured in the air expelled through eructation by cows during milking. This technique can be implemented in large-scale on-farm measurements from dairy cows [242]. Also the sulfur hexafluoride (SF6) tracer technique is a frequently used method to determine the daily methane emission levels of individual cattle, and correlates with values obtained in open-circuit respiratory chambers [243], though the technique requires intensive cow handling, insertion of a rumen bolus and a gas-collection equipment attached to the head.

1.5 GENERAL AIMS

Methane production by ruminants is an unintentional by-product of fermentation and contributes to greenhouse gas emissions. Cross-contamination of feed causes livestock to become unintentionally exposed to subtherapeutic concentrations of antibiotic compounds, aiding to the spread of antibiotic resistance. Both these problems originate from the gastro-intestinal (and rumen) microbial ecosystem of livestock animals. A thorough understanding of the microbial ecosystem of the gastrointestinal tract is essential in these studies and requires the implementation of specific microbial and molecular techniques. The objective of this doctoral research was to implement next generation sequencing techniques in the study of gastro-intestinal microbial communities of farm animals. In the first section, metabarcoding was applied to investigate which factors shape the rumen microbial community composition and activity. In the second section, *in vivo* and *in vitro* experiments were used to investigate if cross-contamination with doxycycline has the potential to enrich resistance genes and resistant species and/or affect the microbial community composition and activity.

The specific aims of this doctoral thesis were as follows:

Section 1

- Develop and compare protocols for the specific sampling of the three rumen environments: fluid, solid and the epithelium. Determine the microbial community composition and identify bacterial and methanogen species that are specifically associated with one of these environments (Chapter 2).
- Investigate to what extent breed-specific factors determine the rumen microbial community and methane emissions by comparing the bacterial and methanogen composition and methane production in the rumen in Holstein-Friesian and Belgian Blue heifers (Chapter 3).
- Investigate to what extent host-specific factors influence the rumen microbial community composition and activity (by VFA and CH_4 production), following a complete rumen content transfer between cows (Chapter 4).

Section 2

- Investigate the effect of feeding pigs 3% of a therapeutic dose of doxycycline (representing a cross-contamination event) on the microbial community

composition and the abundances of specific tetracycline resistance genes in manure of treated pigs (Chapter 5).

- Investigating the effect of 1, 4 (i.e. intestinal concentrations when pigs are exposed to 3% of a therapeutic dose) and 16 mg kg^{-1} doxycycline on the community composition, microbial activity, specific tetracycline resistance genes and abundances of specific resistant species in the microbial community of the *in vitro* simulated pig cecum (Chapter 6). A second part of this Chapter was devoted to evaluating the appropriateness of the *in vitro* model as a simulation of the pig's cecal microbial ecosystem by comparing the *in vitro* microbial community and activity with its *in vivo* counterpart.

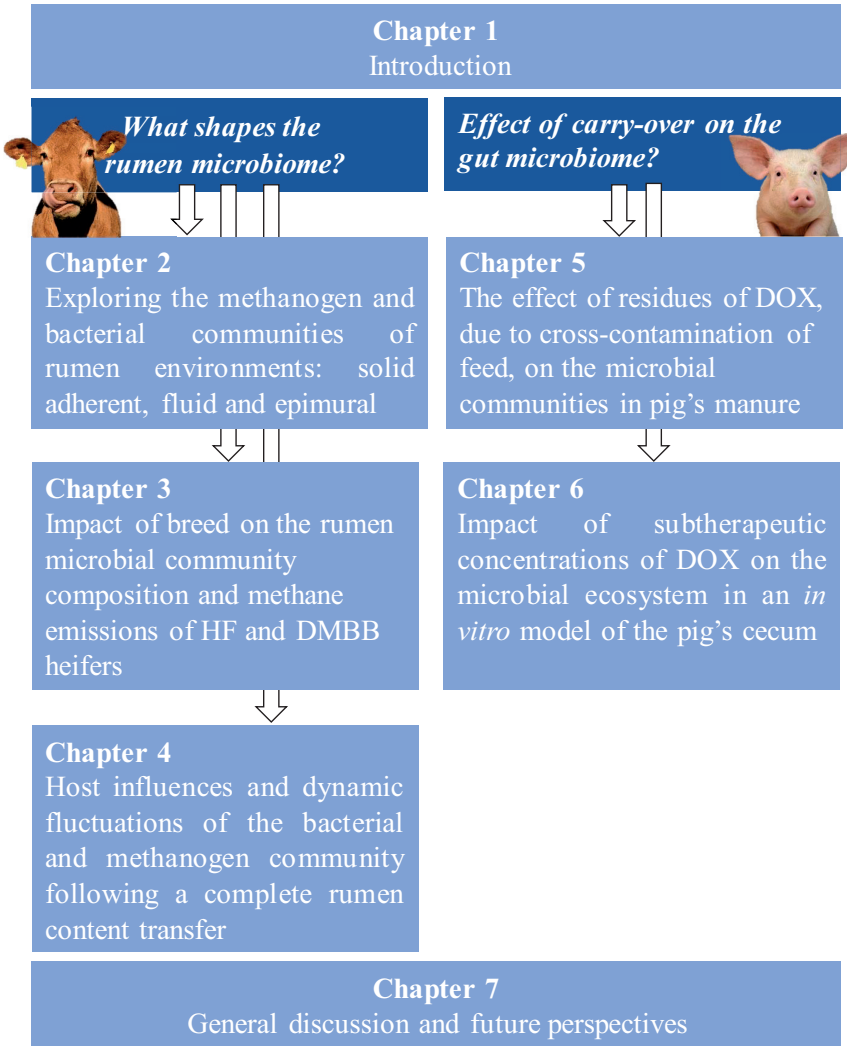


Figure 1.9 Schematic overview of the chapters and content of this PhD dissertation.

Chapter 2

Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural

The content of this chapter is based on:

T. De Mulder, K. Goossens, N. Peiren, L. Vandaele, A. Haegeman, C. De Tender, T. Ruttink, T. Van de Wiele, S. De Campeneere (2016). Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural, FEMS Microbiol. Ecol. doi:10.1093/femsec/fiw25

CHAPTER 2 EXPLORING THE METHANOGEN AND BACTERIAL COMMUNITIES OF RUMEN ENVIRONMENTS: SOLID ADHERENT, FLUID AND EPIMURAL

Abstract

The rumen microbiome occupies a central role in animal health and productivity. A better understanding of the rumen ecosystem is essential to increase productivity or decrease methane production. Samples were collected from the three main rumen environments: the solid-adherent fraction, the liquid fraction and the epithelium. For the liquid and solid fraction, two alternative sample processing protocols were compared, resulting in a total of five sample types: crude solids (S), the eluted solid-adherent fraction (Ad), free-living species in the crude rumen liquid (CRL), strained liquid samples (Lq) and epimural scrapings (Ep). The bacterial and methanogen communities of these sample types were analyzed using 16S metabarcoding and qPCR. The results indicate that the liquid and solid-adherent environments are distinguished mainly by the differential abundance of specific taxonomic groups. Cellulolytic bacteria that pioneer biofilm formation, together with secondary colonizers are prevalent in solid-adherent samples, while dominant species in the fluid samples are primarily identified as consumers of soluble nutrients. Also, methanogen species are found to have a preference for either a solid-adherent or free-living occurrence. The epimural environment is characterized by a different microbial profile. Ten bacterial families and two methanogen genera are almost exclusively found in this environment.

2.1 INTRODUCTION

The complex rumen microbial ecosystem is extensively studied because of the importance of its capacity to convert non-edible feed into human edible food. The rumen functions as a bioconversion "engine", converting cellulose and hemi-cellulose from fibrous feeds into readily available energy sources for the host. In this process, the microbial community occupies a central role as labour force, with each group of species fulfilling a specific niche of the ecosystem. Feed particles enter the reticulorumen through the oesophageal orifice and remain retained in the rumen for prolonged periods. Free-suspended cellulolytic bacteria in the liquid bulk can adhere to the fibrous substrate via the bacterial glycocalyx, adhesins or ligand formations (reviewed by [53]). The attached pioneer species proliferate and encapsulate themselves in extracellular polymeric substances [118]. Cellulose, hemi-cellulose and other macropolymers fuel the metabolic activity in the biofilm and are converted to bacterial biomass and soluble intermediates (sugars, peptides and amino acids), attracting secondary colonizers (reviewed by [111]). Each feed particle can be considered a distinct micro-environment, populated by a unique microbial community. The composition is presumably determined by the duration of incubation, the feed component (i.e. the nutrient availability) and the microbial composition of the liquid surrounding the substrate. Within the rumen ecosystem, three environments can be distinguished: a solid adherent fraction, the liquid fraction and the rumen epithelium. The solid adherent environment is characterised by a complex and divers bacterial community [125], playing the most important role in rumen digestion [244]. The free-living species in the liquid fraction contribute little to the metabolic activity, but rather serve as a relay of bacteria from the solid adherent biofilms to newly ingested feed [126]. Although these liquid and solid environments differ in terms of microbial composition [125] and physical-chemical properties, it is clear that they are prone to continuous interaction and mutual influences.

The rumen epithelium, the third type of environment in the rumen ecosystem, harbours "epimural" bacteria. Because of their close proximity to the host, these species execute a variety of functions that have the potential to significantly modulate host health. They are involved in oxygen scavenging [127], hydrolysis of urea [128] and recycling of epithelial tissue [129].

The methanogen community occupies a central role in the metabolic activities in the rumen. Piao (2014) used pyrosequencing to follow the formation of the adherent communities on switch grass incubated in the rumen. The majority of degradation appeared in the first 30 min of incubation, followed by an increased abundance of adherent methanogens. FISH-CLSM analysis showed that methanoarchaea appear as ball-shaped colonies in the middle of mature

biofilms on microcrystalline cellulose [131]. Methanogens are also found free-living in the rumen fluid, associated as ecto- and endosymbionts of protozoa and attached to the rumen wall [98].

The rumen content is commonly studied by sampling material that represents the rumen bulk, although some researchers make a distinction between the solid and the liquid environments by including fractioned samples in their studies [246–248]. As each fraction of the rumen represents a different environment, the aims of this study were to evaluate the bacterial and methanogen diversity and taxonomic composition of the three main rumen environments: the solid adherent fraction, the fluid (i.e. the liquid fraction containing the free-living species) and the epimural fraction. We analysed this in four cows with the same diet composition, feed intake and lactation stage to account for possible between-animal variation. 16S metabarcoding was used to characterise the community profiles and identify taxonomic groups that are significantly more abundant in specific environments. The biological and biochemical processes that take place in the different environments are described based on known functionalities of the most abundant taxonomic groups. Complementary to metabarcoding, which only determines the relative abundances of the taxonomic groups, qPCR assays for bacteria and methanogens were used to study differences in the bacterial and methanogen load of the three types of rumen environments.

2.2 MATERIAL AND METHODS

2.2.1 Animals, diets and sampling techniques

Four rumen-cannulated Holstein-Friesian dairy cows in mid-lactation were fed a diet with a forage-to-concentrate ratio of 70:30 on dry matter base. The forage was a mixture of prewilted grass and maize silage in the ratio of 58:42 on dry matter base, complemented with balanced concentrate (69%), rumen protected soybean meal (30%) and feed urea (1%). The four cows, representing four biological replicates, were sampled on the same day, two hours after providing morning ration. Samples were collected to represent the three environments of the rumen ecosystem: the liquid fraction, the solid fraction, and the rumen epithelium.

First, rumen fluid was collected through the cannula using a vacuum suction pump connected to a metal perforated sampling probe. To increase representativeness of the sample, the probe was replaced several times to collect rumen fluid from different regions in the rumen. The fluid samples were immediately stored on ice in sealed Erlenmeyers. The rumen fluid was further processed to generate the CRL and Lq sample types. Crude rumen liquid samples (CRL)

were collected by transferring 500 µl of crude rumen liquid directly to a cryovial. These samples consisted of rumen fluid and a minor fraction of small and degraded fibers. To obtain a more thorough separation of the liquid and solid fraction, the protocol from Makkar and McSweeney (2005) was adopted with minor modifications. Rumen liquid was strained through 4 layers of cheesecloth to remove fibers. 60 ml of strained liquid was centrifuged for 10 min at 16.000 x g (4°C). The pellet was washed and resuspended in 20 ml HiTE buffer (50 mM tris-HCl, 5 mM EDTA, pH 8). A portion of 500 µl of the liquid fraction sample (Lq) was transferred to a cryovial.

Second, the solid fraction was sampled by taking rumen fibers through the cannula and immediately placing them on ice in closed plastic containers. Solid samples (S) were collected by squeeze-drying the rumen fibers in four layers of cheesecloth to remove rumen fluid, washing the solids in PBS (Oxoid) and again squeeze-drying the solids in four layers of cheesecloth to remove the PBS. Using forceps, a 0.5 g sample of the solids (S) was transferred to a cryovial. To eluate prokaryotes adherently attached to plant particles, 30 g of rumen solids were squeezed dry in four layers of cheesecloth, washed with PBS, submerged in 80 ml of elution buffer, briefly vortexed and incubated on ice for 2 h to elute the adherent prokaryotes from the plant particles. The elution buffer-fiber suspension was centrifuged for 15 min at 500 x g (4°C) and the supernatant containing the eluted bacteria was transferred to a new centrifuge bottle and centrifuged for 10 min at 16.000 x g (4°C). The pellet was washed and resuspended in 20 ml HiTE buffer. 500 µl of the solid adherent fraction sample (Ad) was transferred to a cryovial. To obtain samples of the epimural fraction (Ep), the rumen content was removed and the rumen wall was rinsed with sterile saline solution at 37°C (0.9% w/v NaCl solution; autoclaved). Epimural samples were collected by scraping the rumen epithelium with a sterile curette and transferring the content to a cryovial. Prior to DNA extraction, all samples were stored at -80°C.

2.2.2 DNA extraction

DNA extraction was performed with the repeated bead beating and column (RBB+C) protocol, as described in [250], with minor modifications. Cells were ruptured in a FastPrep®-24 (MP Biomedicals) (two times 45 s, 6 m s^{-1}) using 0.4 g autoclaved zirconia beads (⌀ = 0.1 mm) and in the presence of a lysis buffer, adopted from [251]. Further extraction steps were carried out as described in [250]. DNA integrity and quantity was subsequently measured with 1.5%

agarose gel electrophoresis, Nanodrop (NanoDrop ND-1000, Thermo Scientific) and the Quantus double-stranded DNA assay (Promega).

2.2.3 16S rRNA amplicon sequencing and data processing

Amplicon sequencing of the bacterial V3-V4 and the methanogen V6-V8 variable region of the 16S rRNA gene was done on 20 samples (n = 4 for each sample type). Preparation of the amplicons was based on the Illumina 16S sequencing library preparation protocol [252], with minor adaptations. The primer pair (Table 2.1) for specific amplification of the V6-V8 region of methanogen 16S rRNA was adopted from [253], with an annealing temperature of 61°C. The amplicon PCR and index PCR were followed by amplicon purification with the CleanPCR reagent kit (CleanNA). Quality control of the final library was performed on the Qiaxcel Advanced using the Qiaxcel DNA High Resolution kit (Qiagen) and the concentration was measured using the Quantus™ double-stranded DNA assay. The final barcoded libraries of each sample were diluted to 10 nM and sequenced using Illumina MiSeq V3-technology (2 x 300bp) by Macrogen. The raw sequence data is stored in the NCBI Short Read Archive (SRA), accession number SRP074884.

The amplicon sequencing dataset was demultiplexed by the sequence provider and barcodes were clipped off. Primer sequences were removed using Trimmomatic v0.32 [254]. Different programs of the Usearch software v7.0.1090 [255] were used, in combination with software packages PEAR and QIIME. For the bacterial dataset, the forward and reverse reads were merged using a minimum overlap length of 120 bp, a minimum and maximum resulting length of 400 bp and 450 bp and a quality threshold of 30 with a minimum length of 200 bp after trimming, using PEAR 0.9.8 [256]. For the methanogen dataset, PEAR was used with different parameter values: a minimum overlap length of 85 bp and a minimum and maximum resulting length of 430 bp and 470 bp. The resulting sequences were further processed using different programs of the Usearch software. Quality filtering was done using “fastq_filter” with a maximum expected error of 3. Next, sequences of all samples that needed to be compared were concatenated, dereplicated (“derep_fulllength”) and sorted by size (“sortbysize”). Uparse (“cluster_otus”) was used to cluster the reads into Operational Taxonomic Units (OTUs) at 97% identity level [257]. Chimeras were removed using Uchime (“uchime_ref”) with the RDP Gold database as a reference [258]. Finally, sequences of individual samples were mapped back to the representative OTUs using the “usearch_global” algorithm (97% identity) and converted to an OTU table using “biom convert” [259]. This procedure resulted in an average of 57 524

reads per sample, with an average read length of 417 bp for the bacterial dataset and an average of 33 353 reads per sample, with an average read length of 451 bp for the methanogen dataset. Resulting OTU tables were annotated with the QIIME software package (v1.8.0) [260]. Representative OTU sequences were aligned to the Greengenes 97% core OTU set (v13_8) [261] for the bacterial dataset and to the RIM database [105] for the methanogen dataset, with a minimum percent identity of 97% using the PyNast algorithm [262] with QIIME default parameters.

2.2.4 Downstream data analysis and statistics

Rarefaction analyses (Figure S2.1) were done using the R-package Vegan [263], indicating that a sequencing depth of 20 000 reads is sufficient to analyse the bacterial communities of the rumen samples. Rarefaction analysis was not done for the methanogen dataset as these communities consisted of a maximum of only 17 OTUs. Shannon-Wiener diversity, transformed Simpson diversity (1-D) indices and observed richness were calculated with the Phyloseq package in R [264]. For subsequent data analysis, only OTUs representing at least 0.1% of the total community in at least one sample were retained thus reducing the total number of OTUs from 1886 to 560. Differences in richness and diversity between the sample types of the liquid and solid environments (Lq, CRL, Ad, S) were analyzed using a linear mixed-effects model [265] including “sample type” as fixed effect and “cow” as random effect, with Tukey-adjustment for post-hoc testing. This statistical model was also used to determine significant differences between relative abundances of each phylum and the major families found in the dataset. The linear model was evaluated by checking if the residuals follow a normal distribution and p-values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure [266]. The epimural samples have a considerably higher within-group variance compared to the other sample types. Because this does not fit the assumption of equal between-group variance required for the linear-mixed effects model analysis, the epimural samples (Ep) were excluded from statistical analysis. A heatmap of the OTU table was generated using the R-package gplots and the heatmap.2 function, using Manhattan distances and UPGMA (unweighted pair group method with arithmetic mean) for hierarchical clustering. Multivariate analysis of the bacterial and methanogen datasets was done using the R package Vegan as described in [267]. The betadisper function was used to ascertain the multivariate spread of the data. If multivariate homogeneity of group dispersions was fulfilled, differences between communities from the five sample types were analyzed by PERMANOVA analysis,

using the adonis function. These significances were further visualized by constructing non-Metric Multidimensional Scaling (nMDS) plots, using Bray−Curtis dissimilarity indices.

2.2.5 Quantitative PCR

QPCR analysis was done on a LightCycler® 480 Real-time PCR System (Roche) using SYBR Green technology. Duplicate samples of a 100-fold dilution of the DNA-extracts were analysed for the abundance of total methanogens, of the orders of the *Methanobacteriales* and *Methanomicrobiales* and the Methanomassiliicoccales. A 1000-fold dilution of the DNA extracts were analysed to quantify the total abundance of bacteria. The primers and PCR conditions are listed in Table 2.1. Each 20 µl reaction mixture contained; 10 µl GoTaq® qPCR Master Mix (Promega), 7.5 pmol of each primer and 5 µl of template DNA. The PCR was carried out in a two-step thermal cycling process that consisted of a hot start activation step of 10 min at 94°C, followed by 40 cycles of 15 s at 95°C and 1 min at the respective annealing temperature (Table 2.1). Melting curve analysis was conducted over a range of 60°C to 95°C in steps of 0.3°C s^{-1} to assess specificity of the amplification products. Within each run, a standard curve was constructed using a 10-fold dilution series of plasmid or gBlock DNA (IDT), containing a strain specific sequence, in order to determine the PCR-efficiency. The total number of gene copies was calculated by converting the quantification cycle values (Cq) to gene copy abundances, taking the PCR efficiency into account. The qPCR

Table 2.1 Primers used in this study

Primer pair	target	purpose	Sequence (5' – 3')	T_a (°C)	reference
AB344_F AB806_R	V3-V4 16S rRNA Bacteria	NGS	CCT ACG GGN GGC WGC AG GAC TAC HVG GGT ATC TAA TCC	55°C	[268]
Ar915_F Ar1386_R	V6-V8 16S rRNA Archaea	NGS	AGG AAT TGG CGG GGG AGC AC GCG GTG TGT GCA AGG AGC	61°C	[253]
Bac338_F Bac518_R	16S rRNA Bacteria	qPCR	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	60°C	[269]
Met630_F Met803_R	16S rRNA total methanogens	qPCR	GGA TTA GAT ACC CSG GTA GT GTT GAR TCC AAT TAA ACC GCA	60°C	[270]
MBT857_F MBT1196_R	16S rRNA *Methanobacteriales*	qPCR	CGW AGG GAA GCT GTT AAG T TAC CGT CGT CCA CTC CTT	60°C	[271]
MMB282_F MMB832_R	16S rRNA *Methanomicrobiales*	qPCR	ATC GRT ACG GGT TGT GGG CAC CTA ACG CRC ATH GTT TAC	60°C	[271]
RCC762_F RCC1099_R	16S rRNA RCC	qPCR	GAC GAA GCC CTG GGT C GAG GGT CTC GTT CGT TAT	60°C	[272]
qmcrA_F qmcrA_R	mcrA total methanogens	qPCR	TTC GGT GGA TCD CAR AGR GC GBA RGT CGW AWC CGT AGA ATC C	60°C	[273]

results are displayed in two ways: either expressed as number of gene copies per ng DNA in 5 µl starting volume of the PCR, or relative to the total abundance of bacterial 16S gene copies in the sample.

2.3 RESULTS

2.3.1 Bacterial community structure of the three environments

The bacterial community structure of the three main rumen environments was investigated: the liquid environment (Lq, CRL), the solid adherent environment (S, Ad) and the rumen epimural environment (Ep). The observed number of OTUs varies between cows and sample types, while the Shannon-Wiener and Simpson diversity indices of the solid adherent environment (S and Ad) are significantly ($p < 0.05$) higher than that of the liquid environment (Lq and CRL) (Figure 2.1). The diversity indices of the four epimural samples varies greatly. The epimural sample from cow 4 stands out as it has the lowest diversity while the epimural sample from cow 3 has both the highest richness and diversity.

The nMDS profile (Figure 2.2) provides further insight in the differences of the bacterial community structure that are observed between the environments. PERMANOVA analysis of Bray−Curtis dissimilarity indices ($p < 0.001$) confirms the separation of environmental community structures. The high variance between Ep samples is visible as the large distances between the four Ep samples. The Lq and CRL samples of the four independent cows cluster together, whereas the Ad and S samples cluster separately from each other.

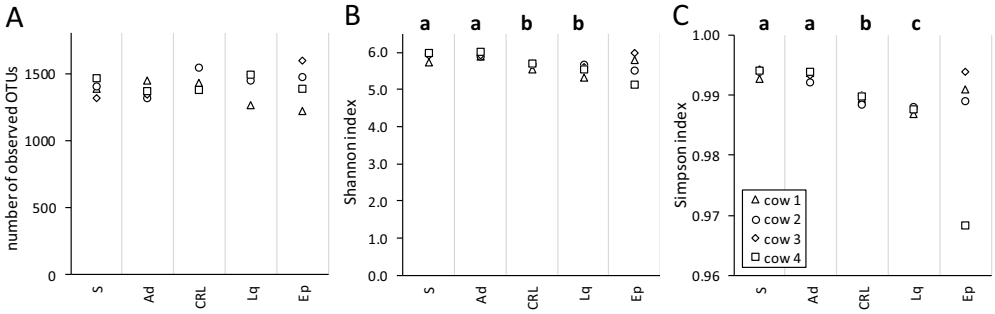


Figure 2.1 Graphical representation of **[A]** richness (observed OTUs), **[B]** Shannon diversity and **[C]** Simpson diversity indices of the bacterial communities of five sample types collected from four cannulated cows. The letters (a, b, c) indicate the statistical classification in homogeneous groups based on a linear mixed-effects model. Sample types without a common letter are significantly different ($p < 0.05$).

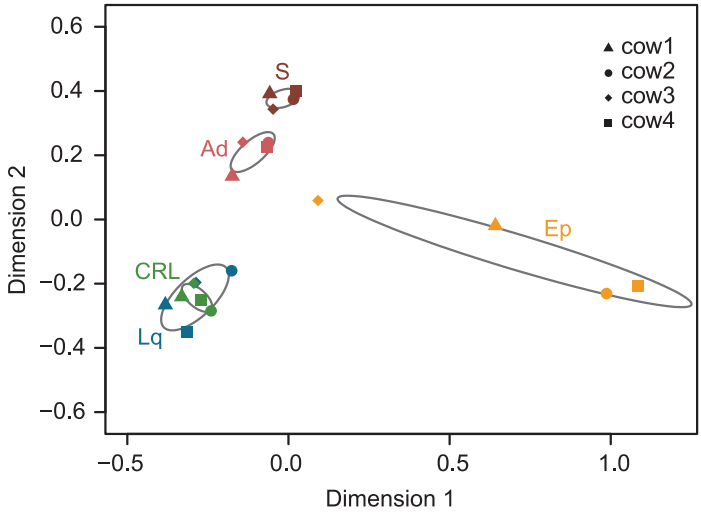


Figure 2.2 NMDS profile of pairwise community dissimilarity (Bray-Curtis) indices of bacterial 16S sequencing data from five sample types collected from four cows.

The hierarchically clustered heatmap (Figure2.3) confirms the observations of the nMDS plot. The samples form clusters that are consistent with the grouping according to sample type and environment, except for the Ep sample of cow 3, which clusters with the solid adherent samples. The high similarity in community composition of the epimural sample of cow 3 to that of the solid communities, together with the high number of observed OTUs and the higher diversity, suggest that remainders of solid fibers attached to the rumen wall were contaminating this sample. Therefore, this sample was excluded from further taxonomic analysis. The rumen fluid (Lq and CRL) contained a bacterial population that is distinct from the solid adherent and epimural communities. The average relative abundances for each phylum and the major families are summarized in Table 2.2.

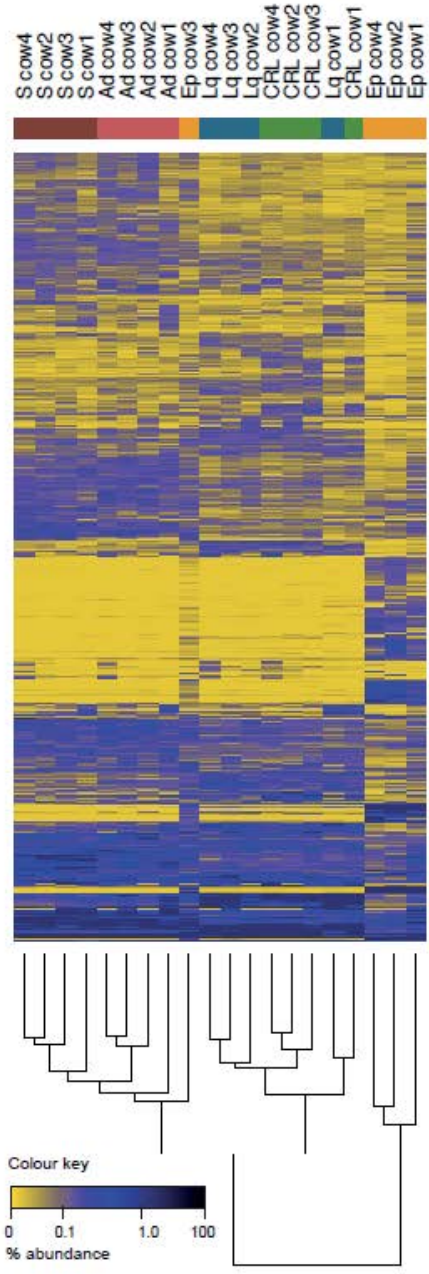


Figure 2.3 Heatmap of the bacterial OTUs. The dendrogram indicates the community resemblance between samples based upon UPGMA clustering and Manhattan distance method. The color code in the side bar indicates the sample types: S, Ad, CRL, Lq and Ep.

2.3.2 Differences between the communities in epimural and the solid and liquid environments

The phyla of *Bacteroidetes*, *Firmicutes* and *Proteobacteria* account for 90% of the epimural community. The *Firmicutes* was the abundant phylum in each rumen environment, including the epimural, and was even the most abundant in Ep samples as compared to the other sample types. Notably, the *Bacteroidetes* remained the second largest phylum, but was considerably less represented in the epimural environment, whereas the phylum of *Proteobacteria* was more abundant in the epimural samples than in liquid and solid samples (Table 2.2). The candidate phylum *BD1-5* contributes, on average, 1.7% to the epimural communities but was barely detected in the other environments. Within the phyla of *Proteobacteria*, *Firmicutes* and *Actinobacteria*, 10 families were identified with high relative abundances in the Ep samples but were virtually undetected or detected at much lower relative abundances in the other sample types. Detailed analysis at deeper taxonomic levels revealed 92 OTUs that were at least ten times more abundant in epimural samples than in the rumen bulk (i.e. the rumen fluid samples: Lq and CRL and the solid adherent samples: Ad and S).

Table 2.2 Overview of the average relative abundance (%) and standard deviation of the bacterial phyla and major families in the five sample types collected from four cannulated cows. The superscript letters indicate the statistical classification in homogeneous abundance groups based on a linear mixed-effects model. Sample types without a common superscript are significantly different ($p < 0.05$). The sample type(s) where the taxonomic group is most abundant is indicated in green.

Taxonomy	Solid adherent environment		Liquid environment		epimural
	S	Ad	CRL	Lq	Ep
Firmicutes	**44.0 ± 1.70^{a}**	**40.3 ± 0.55^{a}**	**27.5 ± 2.23^{b}**	**30.7 ± 2.17^{b}**	**46.6 ± 5.24**
Lachnospiraceae	18.4 ± 1.57^{a}	16.0 ± 1.37^{b}	9.01 ± 1.66^{c}	10.8 ± 1.43^{c}	20.5 ± 2.39
Ruminococcaceae	16.0 ± 0.75^{a}	14.1 ± 1.33^{a}	10.7 ± 1.74^{b}	10.4 ± 1.33^{b}	7.87 ± 1.37
Clostridiales Family XIII	0.59 ± 0.13^{ab}	0.70 ± 0.14^{a}	0.29 ± 0.03^{c}	0.41 ± 0.11^{bc}	8.89 ± 2.63
Christensenellaceae	4.10 ± 0.49^{a}	3.07 ± 0.56^{b}	2.27 ± 0.11^{c}	2.77 ± 0.21^{bc}	3.71 ± 0.71
Acidaminococcaceae	3.98 ± 0.46^{b}	5.24 ± 0.29^{a}	3.84 ± 0.44^{b}	4.08 ± 0.6^{ab}	2.86 ± 0.75
Erysipelotrichaceae	0.47 ± 0.10^{ab}	0.50 ± 0.18^{a}	0.37 ± 0.13^{bc}	0.31 ± 0.1^{c}	1.50 ± 0.19
Defluviitaleaceae	0.10 ± 0.04^{ab}	0.14 ± 0.02^{a}	0.07 ± 0.02^{b}	0.08 ± 0.02^{b}	1.20 ± 0.27
Veillonellaceae	0.36 ± 0.10^{c}	0.61 ± 0.34^{bc}	0.91 ± 0.35^{b}	1.82 ± 0.61^{a}	0.13 ± 0.02
Bacteroidetes	**40.4 ± 1.18^{d}**	**44.6 ± 1.28^{c}**	**55.9 ± 1.66^{a}**	**50.0 ± 2.42^{b}**	**26.3 ± 3.62**
Prevotellaceae	26.3 ± 1.5^{b}	27.7 ± 3.62^{b}	41.8 ± 2.09^{a}	39.2 ± 4.16^{a}	13.4 ± 3.37
Rikenellaceae	5.57 ± 0.76^{a}	6.44 ± 1.52^{a}	3.76 ± 0.68^{b}	3.59 ± 0.78^{b}	6.71 ± 1.00
Bacteroidales BS11 gut group	1.86 ± 0.57^{b}	2.68 ± 0.75^{a}	2.03 ± 0.34^{ab}	1.90 ± 0.39^{b}	2.81 ± 0.04

Bacteroidales S24-7	5.01 ± 0.61^{a}	5.70 ± 0.28^{a}	2.34 ± 0.69^{b}	2.73 ± 0.69^{b}	1.77 ± 0.72
Bacteroidales RF16	0.44 ± 0.07^{b}	0.56 ± 0.03^{b}	3.91 ± 0.86^{a}	1.63 ± 0.48^{b}	0.59 ± 0.231
Bacteroidaceae	0.23 ± 0.09^{a}	0.29 ± 0.08^{a}	0.12 ± 0.03^{b}	0.13 ± 0.07^{b}	0.16 ± 0.03
Fibrobacteres *Fibrobacteraceae*	**4.25 ± 0.88^{a}**	**2.42 ± 0.34^{b}**	**2.11 ± 0.31^{b}**	**1.77 ± 0.47^{b}**	**2.33 ± 1.41**
Proteobacteria	**2.59 ± 0.75^{d}**	**5.65 ± 1.23^{c}**	**8.17 ± 1.59^{b}**	**10.6 ± 1.07^{a}**	**16.3 ± 6.05**
Cardiobacteriaceae	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	9.49 ± 5.49
Comamonadaceae	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	2.68 ± 0.83
Desulfobulbaceae	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	2.29 ± 0.27
Succinivibrionaceae	2.09 ± 0.64^{d}	4.82 ± 1.14^{c}	7.65 ± 1.43^{b}	9.96 ± 0.97^{a}	0.50 ± 0.02
Campylobacteraceae	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.99 ± 0.29
Neisseriaceae	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.01
Desulfovibrionaceae	0.31 ± 0.08^{b}	0.62 ± 0.12^{a}	0.18 ± 0.03^{b}	0.19 ± 0.06^{b}	0.18 ± 0.03
Actinobacteria	**1.54 ± 1.48**	**0.84 ± 0.43**	**1.46 ± 0.75**	**1.30 ± 0.69**	**1.50 ± 0.30**
Coriobacteriaceae	0.20 ± 0.08^{b}	0.35 ± 0.03^{a}	0.18 ± 0.08^{b}	0.33 ± 0.19^{ab}	0.51 ± 0.13
Actinomycetaceae	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.54 ± 0.65
Bifidobacteriaceae	1.34 ± 1.51	0.49 ± 0.43	1.28 ± 0.71	0.96 ± 0.68	0.45 ± 0.33
Candidate division TM7	**1.56 ± 0.29^{a}**	**1.30 ± 0.30^{ab}**	**1.20 ± 0.27^{b}**	**1.21 ± 0.28^{b}**	**1.57 ± 0.48**
Spirochaetes *Spirochaetaceae*	**2.57 ± 0.35^{a}**	**1.87 ± 0.50^{ba}**	**0.73 ± 0.08^{c}**	**1.07 ± 0.19^{bc}**	**1.11 ± 0.81**
Cyanobacteria	**0.42 ± 0.14^{b}**	**0.32 ± 0.11^{b}**	**0.94 ± 0.43^{a}**	**0.99 ± 0.4^{a}**	**0.10 ± 0.04**
Chloroplast	0.17 ± 0.08^{a}	0.01 ± 0.00^{b}	0.02 ± 0.01^{b}	0.01 ± 0.01^{b}	0.03 ± 0.01
(Class) *Melainabacteria* (Order) *Gastranaerophilales*	0.25 ± 0.09^{c}	0.31 ± 0.12^{bc}	0.92 ± 0.44^{a}	0.98 ± 0.42^{a}	0.07 ± 0.02
Lentisphaera	**0.25 ± 0.10^{c}**	**0.42 ± 0.11^{bc}**	**0.83 ± 0.13^{a}**	**0.47 ± 0.18^{b}**	**0.07 ± 0.04**
RFP12 gut group	0.22 ± 0.09^{b}	0.36 ± 0.10^{b}	0.70 ± 0.07^{a}	0.41 ± 0.16^{b}	0.05 ± 0.03
Victivallaceae	0.03 ± 0.02^{b}	0.07 ± 0.02^{b}	0.14 ± 0.06^{a}	0.06 ± 0.03^{b}	0.02 ± 0.01
Tenericutes	**0.32 ± 0.02^{c}**	**0.47 ± 0.19^{bc}**	**0.74 ± 0.08^{a}**	**0.55 ± 0.06^{ab}**	**0.13 ± 0.03**
(Class) *Mollicutes* *Anaeroplasmataceae*	0.17 ± 0.05^{b}	0.33 ± 0.23^{ab}	0.50 ± 0.14^{a}	0.34 ± 0.12^{ab}	0.09 ± 0.03
(Order) RF9	0.16 ± 0.06^{ab}	0.14 ± 0.05^{b}	0.24 ± 0.12^{a}	0.21 ± 0.07^{ab}	0.04 ± 0.01
Candidate division SR1	**0.59 ± 0.27**	**0.63 ± 0.22**	**0.64 ± 0.24**	**0.69 ± 0.21**	**0.55 ± 0.16**
Chloroflexi *Anaerolineaceae*	**0.40 ± 0.05^{a}**	**0.37 ± 0.12^{a}**	**0.15 ± 0.02^{b}**	**0.21 ± 0.06^{b}**	**0.34 ± 0.06**
Synergistetes *Synergistaceae*	**0.14 ± 0.04^{b}**	**0.25 ± 0.05^{a}**	**0.10 ± 0.02^{bc}**	**0.07 ± 0.03^{c}**	**0.42 ± 0.03**
Elusomicrobia	**0.07 ± 0.03**	**0.08 ± 0.04**	**0.06 ± 0.05**	**0.07 ± 0.05**	**0.06 ± 0.01**
Planctomycetes *Planctomycetaceae*	**0.03 ± 0.02**	**0.03 ± 0.02**	**0.04 ± 0.01**	**0.06 ± 0.01**	**0.08 ± 0.04**
BD1-5	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.01 ± .0.00**	**0.01 ± 0.00**	**1.70 ± 0.80**

2.3.3 Methanogen community structure

The methanogen community richness was a hundred fold lower than that of the bacterial communities in the rumen ecosystem. Only seventeen OTUs identified as *Euryarchaeota* were detected across the five sample types, corresponding to 64% of all reads obtained with the methanogen specific 16S V6-V8 primers. Other reads were annotated as Bacteria or remained unassigned and were removed during read processing. Although all samples had a similar richness, ranging between 13 and 17 OTUs per sample, the diversity indices were different between sample types (Figure 2.4). According to the Simpson index, epimural samples showed the lowest diversity and the solid adherent samples the highest ($p < 0.05$). The five sample types from the four different cows form separate clusters on an nMDS plot (Figure 2.5), showing that patterns were consistent across animals. The CRL and Lq samples cluster close together, indicating very similar community structures. In contrast, the epimural and solid based samples display more variation. An overview of the methanogen OTUs and their relative abundances are summarized in Table 2.3. The genus of *Methanobrevibacter* was the most dominant taxonomic group in the rumen (on average, 72.6 ± 7.9% of the community across all samples) and was further subdivided into the *Boviskoreani*, *Gottschalkii* and *Ruminantium* clades. The relative abundance of the *Mbb. Gottschalkii* clade was higher in the epimural and liquid fraction than in the solid adherent fraction. On the other hand, the *Mbb. Ruminantium* clade is significantly more abundant in the solid adherent fraction than in the liquid fraction, and also significantly more abundant in the S samples as compared to the Ad samples. Furthermore, the

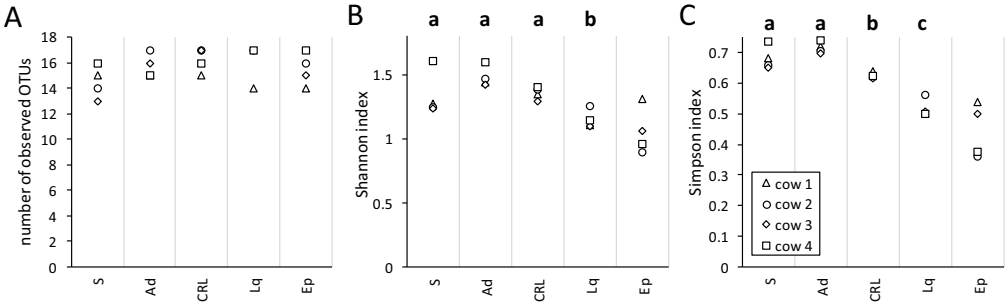


Figure 2.4 Graphical representation of the **[A]** richness (number of observed OTUs), **[B]** Shannon diversity and **[C]** Simpson diversity indices of the methanogen communities of five sample types collected from four cannulated cows. The letters (a, b, c) indicate the statistical classification in homogeneous groups based on a linear mixed-effects model. Sample types without a common letter are significantly different ($p < 0.05$).

Methanosphaera is significantly more abundant in the S samples as compared to the Ad samples. *Methanobacterium* sp. and *Methanimicrococcus* sp. were almost exclusively detected in epimural samples (Table 2.3). Eight OTUs are assigned to the family of the *Methanomassiliicoccaceae* (also known as Rumen Cluster C; RCC). One OTU, belonging to *Mmc.* Group 12, was the most dominant representative of the family of the *Methanomassiliicoccaceae.* Two OTUs, one belonging to an unidentified group and one belonging to *Mmc*. group 8, have a considerably higher relative abundance in the epimural samples, as compared to the solid and liquid samples.

Table 2.3 Overview of the average relative abundance (%) and standard deviation of methanogen OTUs (with complete identification) in the five sample types collected from four cannulated cows. The superscript letters indicate the statistical classification in homogeneous abundance groups based on a linear mixed-effects model. Sample types without a common superscript are significantly different ($p < 0.05$). The sample type(s) where the taxonomic group is most abundant is indicated in green.

Taxonomy	Solid adherent environment		Liquid environment		epimural
	S	Ad	CRL	Lq	Ep
Methanobacteria*; *Methanobacteriales*; *Methanobacteriaceae					
Methanobacterium alkaliphilum	0.01 ± 0.01	0.01 ± 0.01	0.05 ± 0.02	0.06 ± 0.02	3.51 ± 0.76
Methanobrevibacter boviskoreani clade	0.06 ± 0.04^{b}	0.05 ± 0.03^{b}	0.54 ± 0.34^{ab}	0.58 ± 0.40^{a}	0.04 ± 0.02
Methanobrevibacter gottschalkii clade	42.1 ± 5.63^{c}	41.8 ± 4.40^{c}	56.4 ± 1.44^{b}	67.2 ± 2.08^{a}	74.9 ± 5.86
Methanobrevibacter gottschalkii clade	0.76 ± 0.44^{b}	0.55 ± 0.31^{b}	1.70 ± 0.75^{a}	1.67 ± 0.51^{a}	1.72 ± 0.37
Methanobrevibacter ruminantium clade	31.9 ± 4.38^{a}	20.7 ± 2.81^{b}	13.7 ± 1.28^{bc}	10.2 ± 2.23^{c}	6.52 ± 1.72
Methanosphaera sp. Group5	0.58 ± 0.84	0.84 ± 1.23	0.27 ± 0.44	0.13 ± 0.16	0.14 ± 0.19
Methanosphaera sp. ISO30F5	18.0 ± 2.57^{a}	9.13 ± 0.14^{b}	4.74 ± 0.38^{c}	3.61 ± 1.26^{c}	5.27 ± 0.30
Methanosphaera sp. ISO30F5	0.21 ± 0.10^{a}	0.09 ± 0.06^{b}	0.02 ± 0.01^{b}	0.02 ± 0.02^{b}	0.00 ± 0.00
Methanomicrobia*; *Methanosarcinales*; *Methanosarcinaceae					
Methanimicrococcus blatticola	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.16 ± 0.10
Thermoplasmata*; *Methanomassiliicoccales*; *Methanomassiliicoccaceae					
Unidentified group	0.11 ± 0.07	0.13 ± 0.11	0.29 ± 0.21	0.29 ± 0.17	1.23 ± 0.48
Group 10 sp.	0.68 ± 0.47	1.07 ± 0.39	1.25 ± 0.51	0.91 ± 0.46	1.42 ± 0.31
Group 10 sp.	0.79 ± 0.72	0.70 ± 0.37	0.40 ± 0.21	0.25 ± 0.15	0.05 ± 0.03
Group 10 sp.	0.35 ± 0.59	0.29 ± 0.41	0.16 ± 0.23	0.10 ± 0.13	0.05 ± 0.06
Group 11 sp. BRNA1	0.10 ± 0.07^{c}	1.27 ± 0.48^{a}	0.95 ± 0.15^{ab}	0.65 ± 0.16^{bc}	0.12 ± 0.09
Group 12 sp. ISO40H5	4.03 ± 2.52^{c}	23.1 ± 6.69^{a}	18.6 ± 2.75^{ab}	13.4 ± 1.83^{b}	2.56 ± 1.80
Group 8 sp. WGK1	0.03 ± 0.02^{b}	0.04 ± 0.02^{b}	0.35 ± 0.10^{a}	0.39 ± 0.08^{a}	2.15 ± 1.21
Group 9 sp. ISO40G1	0.29 ± 0.20^{b}	0.31 ± 0.04^{b}	0.64 ± 0.12^{a}	0.53 ± 0.22^{ab}	0.17 ± 0.12

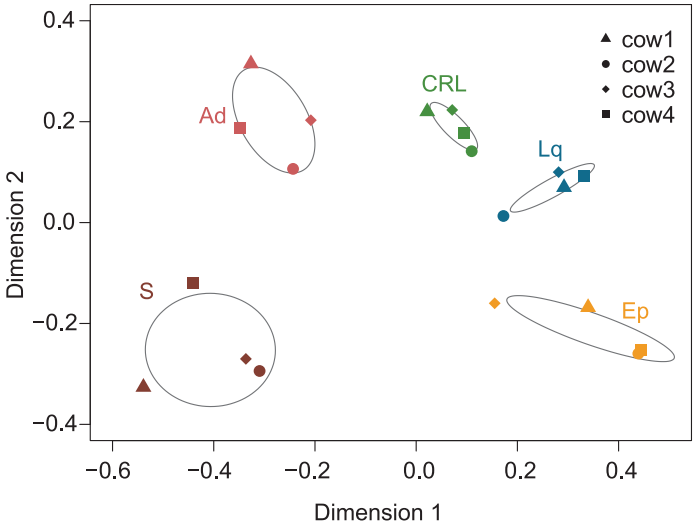


Figure 2.5 NMDS profile of pairwise community dissimilarity (Bray-Curtis) indices of methanogen 16S sequencing data from five sample types collected from four cows.

2.3.4 Absolute quantification

qPCR assays targeting total bacteria, total methanogens, the order of Methanomassiliicoccales, the phylum of *Methanomicrobia,* and the *Methanobacteria*, give an in-depth overview of the bacterial and methanogen community sizes of the different rumen environments. The absolute quantification of bacteria and methanogens is complementary to metabarcoding, which only provides relative abundances. The qPCR measurements (Figure 2.5) indicate that less than 2% of the rumen prokaryotic community consists of methanogen species. In absolute measures, the sample types representing the solid adherent and the liquid fraction have a similar bacterial and methanogen load (on average, 3.43×10^8 and 3.44×10^6 16S copies per ng DNA extract, respectively) (Figure 2.5). In contrast, the epimural samples have lower absolute numbers of bacterial and methanogen 16S rRNA gene copies (on average, 5.58×10^6 and 1.16×10^5 copies per ng DNA extract, respectively), but contain a larger fraction of methanogens, relative to the number of bacteria. *Methanomicrobia* measurements were below the limit of quantification for all samples and were therefore not included in Figure 2.5. Total methanogens were quantified using both 16S methanogen specific primers and primers targeting the mcrA gene, which is exclusively present in methanoarchaea as it encodes for methyl coenzyme M reductase [274]. As this enzyme is a prerequisite for methanogenesis, this gene can be used for specifically

quantifying and identifying methanogens. The absolute quantifications of methanogens (gene copies per ng DNA) using both primer pairs are highly correlated ($R^2 = 0.885$) (Figure S2.2).

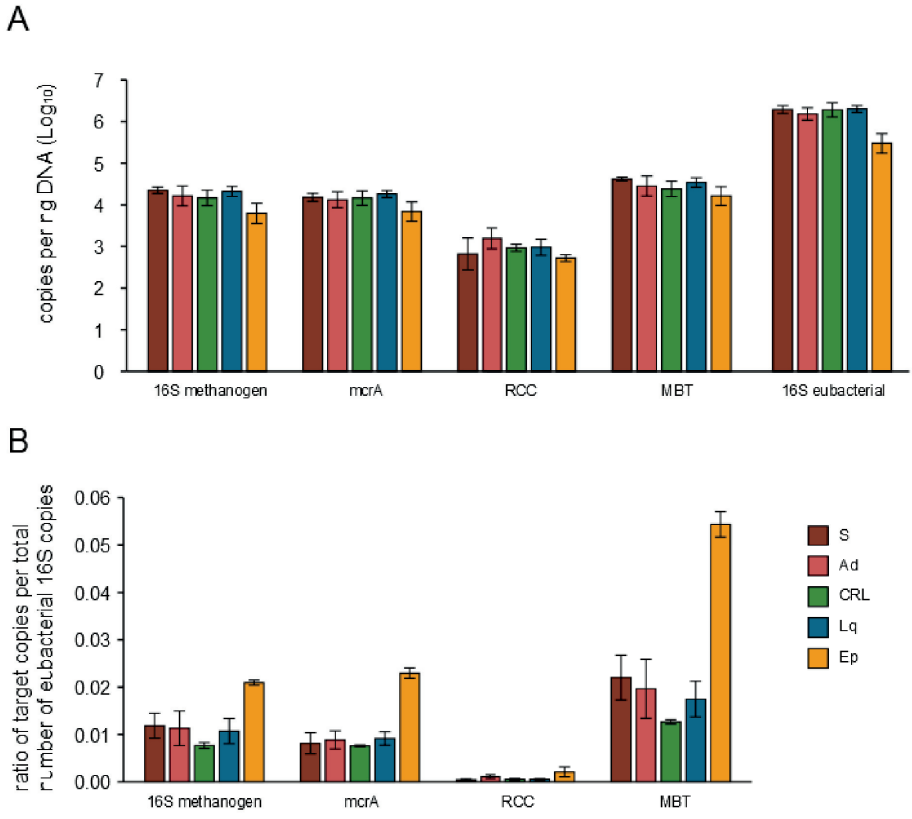


Figure 2.5 Absolute quantification of bacteria, total methanogens (based on mcrA and 16S genes), Methanomassiliicoccales (also known as RCC) and the order of *Methanobrevibacter* (MBT) of five sample types from four cannulated cows: **[A]** absolute gene abundances expressed as $\log_{10}$ gene copies per ng of DNA extract and **[B]** relative gene abundances, normalized to bacterial 16S rRNA gene copies.

2.4 DISCUSSION

The solid adherent (represented by Ad and S samples) and liquid (Lq and CRL samples) environment do not display differences in taxonomic composition, but can be distinguished based on the relative abundance of species. No taxonomic groups were identified that were unique for the liquid or solid environment. This can be expected because solid adherent bacteria or methanogens can eventually end up in the fluid due to declining substrate availability, biofilm dispersion or fiber erosion [126]. The bacterial and methanogen diversity of solid based

samples is significantly higher than that of the fluid based samples, confirming observations of earlier studies using clone libraries [275,276]. All sample types are dominated by the phyla of *Bacteroidetes* and *Firmicutes.* The *Bacteroidetes* are significantly more prevalent in liquid based samples, while the *Firmicutes* are the most abundant in solid based samples, in line with previous publications [277,278]. In our study, the phylum of *Fibrobacteres*, exclusively represented by the genus *Fibrobacter*, is the third largest phylum in S samples, but is significantly less abundant in the Ad samples, in which the *Proteobacteria* is the third largest phylum. The *Fibrobacter* genus is recognized as a major group of lignocellulolytic bacteria. Electron microscopy has shown that *F. succinogenes* can tightly adhere to plant cell walls and form digestive pits [279]. Within the phylum of the *Firmicutes*, the three main families: *Lachnospiraceae*, *Ruminococcaceae* and *Christensenellaceae* are more abundant in the solid based samples than in the liquid, and comprise over 80% of the reads assigned to this phylum. The families *Ruminococcaceae* and *Lachnospiraceae* are known to include cellulolytic and fibrolytic bacteria [280–282]. *Pseudobutyrivibrio* species (*Lachnospiraceae*) isolated from the rumen contain a specialized enzyme system for hemicellulose degradation [283,284] and also *Ruminococcus* species (*Ruminococcaceae*) possess specialized mechanisms for fiber adhesion and cellulose degradation [285,286]. The relative abundances of these genera were found to be significantly higher in S than in Ad samples.

Cellulolytic bacteria are prominent members of solid adherent colonies, initiating and supporting biofilm growth. During maturation of the biofilm, adherent bacteria degrade the fibrous material, causing the biofilm to grow inward and become embedded in the fiber. In the S samples, DNA was extracted directly from the fibrous material using repeated bead beating, chemical lysis and heat treatment. In contrast, during the sample preparation of the Ad samples, the biofilm presumably protects the strongly adherent bacteria from the elution buffer, causing only superficially bound bacteria to be extracted with the Ad samples. This could explain why the relative abundance of the phylum of *Fibrobacteres* is almost twice as high in the S samples compared to Ad samples. Also the *Lachnospiraceae*, *Ruminococcacea* and *Christensenellaceae* have a higher relative abundance in the S samples compared to Ad samples. Interestingly, also the *Spirochaetes*, represented solely by the genus of *Treponema*, has a significantly higher relative abundance in the solid based samples, and with a higher abundance in the S samples than in Ad samples. In an isolation experiment conducted by Kudo, Cheng and Costerton (1987), *Treponema* species were detected during the cultivation of cellulolytic rumen bacteria. *Treponema* species cannot utilize cellulose as a carbon source, but engage in close synergetic

relationships with cellulolytic bacteria like *F. succinogenes* to access the soluble sugars released during fiber degradation [42]. Following this assumption, the higher abundance of *Spirochaetes* in S samples suggests that *Treponema* species are incorporated in biofilms as secondary metabolizers.

Bacteroidetes is one of the most dominant phyla in all sample types, mainly due to the presence of the *Prevotellaceae*, notably the most dominant family in the rumen ecosystem. The *Prevotellaceae* comprises up to 40% of the community in liquid samples. *Prevotella* (isolated from the rumen) are non-cellulolytic but have a broad saccharolytic and proteolytic potential [31]. Co-occurrence of *Prevotella* and cellulolytic bacteria improves digestion of cellulose [62] and plant cell wall protein [287]. The omnipresence and prevailing dominance in rumen environments implies an essential role of *Prevotella* in the metabolic activity of the rumen ecosystem, although the significantly higher abundance in liquid samples suggests a preference for a free-living life style. Also the *Bacteroidales* RF16 group has a significantly higher prevalence in rumen fluid compared to the solid adherent and epimural samples. Other taxonomic groups within the Bacteroidetes, such as the *Rikenellaceae*, the *Bacteroidaceae* and the *Bacteroidales* S24-7 group had a significantly higher relative abundance in the solid adherent samples as compared to the liquid, suggesting a possible contribution of these species to cellulose digestion directly, or indirectly as commensals to cellulolytic bacteria.

Beside the *Prevotellaceae*, other bacterial families with a significantly higher relative abundance in the liquid samples are the *Veillonellaceae* (the phylum *Firmicutes*), *Succinivibrionaceae* (*Proteobacteria*) and the order of *Gastranaerophilales* (unkown family level, order of *Cyanobacteria*). Most of the OTUs of *Succinivibrionaceae* were further annotated to the genera of *Succinimonas*, *Succinivibrio* and *Ruminobacter*. These genera are commonly found at high numbers in ruminant animals and are involved in the degradation of soluble starch [288,289]. The phylum of *Cyanobacteria* in the rumen is otherwise known as the candidatus phylum of *Melainabacteri*a. Despite their original classification as *Cyanobacteria*, *Melainabacteria* are non-photosynthetic and obtain energy through anaerobic fermentation of starch, glycogen, glucose and mannose [290]. The function of *Selenomonas*, a genus within the *Veillonellaceaea*, involves the fermentation of soluble sugars, glycerol and lactate [291]. Co-cultivation experiments further indicated that *Selenomonas ruminantium* is also capable of converting succinate to propionate [45]. The higher abundance of these taxonomic groups in the rumen fluid samples supports the idea that free-living bacteria are involved in the fermentation of soluble carbohydrates and metabolic end products of cellulose digestion. Many

prevalent cellulolytic bacteria in the rumen, like *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*, produce succinate and acetate as a metabolic end product of cellulose fermentation [56]. Succinate does not accumulate in the rumen but rather serves as a precursor of propionate, a major end product of rumen fermentation. Succinate consuming propionate producing bacteria such as *Selenomonas ruminantium* [45] and *Succiniclasticum ruminis* [47] can thrive in the ecosystem by maintaining a close synergetic relationship with active cellulolytic bacteria in the solid adherent biofilms. Both the genus *Succiniclasticum* (the only genus within the *Acidaminococcaceae*) and *Selenomonas* have a notably higher relative abundance in the Ad samples as compared to the S samples, which could indicate that these species are closely associated with solid adherent biofilms as secondary colonizers.

Researchers investigating the effect of diet types, feed alterations or mitigation strategies, often take crude rumen liquid samples for analysis [292–294]. This study indicates that CRL samples provide a good representation of the free-living bacteria and archaea, but they are not representative for the entire rumen ecosystem. The results of this study suggest that the Lq and CRL sample types give a comparable representation of the microbial community. The observed differences in relative abundance of taxonomic groups in the Lq and CRL samples could result from the difference in sample preparation. The preparation of the Lq sample type results in a purification and enrichment of the free-living bacteria. The solid adherent fraction is best represented by the S sample type because the sample processing is better suited to include prominent biofilm members. The Ad sample procedure was developed by [125,249] based on previous studies that suggested a high detachment of solid adherent species by submerging rumen solids in anaerobic saline buffer with tween-80 and under cooled temperature [295,296]. The discrepancies between S and Ad community profiles suggest that the elution protocol resulted in an incomplete recovery of attached bacteria because the proportions of known cellulolytic and therefore solid-adherent bacterial taxa in the Ad samples had a significantly lower relative abundance than in the S samples.

The epimural fraction is distinct from the bacterial communities in the rumen bulk in terms of diversity and community structure. Detailed analysis of the taxonomic profiles reveals obvious differences between the microbial communities of the rumen bulk and the epithelium. Many OTUs exclusively found in epimural samples and annotated to genus level, give more insight into the functionality of the epimural community (Supplementary table 1). *Howardella* ($\bar{x}$ = 1.84 ± 0.90%), with the only known species *H. ureilytica*, has a strong ureolytic activity and presumably occupies a role in the biochemical pathway of urea hydrolysis [297].

Comamonas ($\bar{x}$ = 2.43 ± 0.77%), *Suttonella* ($\bar{x}$ = 8.61 ± 5.06%), and *Desulfobulbus* (5 OTUs, $\bar{x}$ = 2.07 ± 0.26%) are aerobic *Proteobacteria* and thus likely involved in oxygen scavenging. qPCR further indicated that the epimural fraction has a much lower bacterial and methanogen load compared to the rumen bulk samples. However, the methanogen abundance in the epimural environment is twice higher as compared to the other environments.

The methanogen community diversity was greatly underestimated if 16S bacterial primers are used to observe the prokaryotic community of the rumen environments. Less than 2% of the reads were assigned to *Euryarchaeota* and further annotated to only three genera: *Methanobrevibacter*, *Methanosphaera* and *Thermoplasmatales* (data not shown). A better understanding of the true methanogen richness and diversity was obtained using methanogen specific 16S primers. The *Methanobrevibacter* clades *boviskoreani* and *gotschalkii* are significantly higher represented in the liquid samples. The *Methanobrevibacter ruminantium* clade and *Methanosphaera* sp. ISO30F5 have a significantly higher relative abundance in the solid based samples, but amongst those, the relative abundance in S samples is significantly higher than in Ad samples. Similar results were obtained by Henderson et al. (2013) when comparing the solid and liquid fraction. This could indicate that these methanogens make up an intrinsic part of the solid adherent biofilms [298]. *Methanimicrococcus blatticola*, the only methanogen detected from the *Methanomicrobia*, and *Methanobacterium alkaliphilum* are detected in the epimural samples but are virtually undetected in the rumen bulk samples.

2.5 CONCLUSION

Epimural (Ep), crude solids (S) and strained liquid (Lq) samples, individually, give a specific view of the microbial communities of the different environments. The parallel analysis of these three sample types using metabarcoding and qPCR, provides a more complete understanding of the complexity of the rumen ecosystem. The microbial communities of the rumen liquid and the solid adherent fraction display the same taxonomic groups in both environments, suggesting continuous interactions, but with different levels of abundance. The epimural fraction, on the other hand, is characterised by the presence of different taxonomic groups, performing specialized and niche adapted functions.

2.6 SUPPLEMENTARY DATA

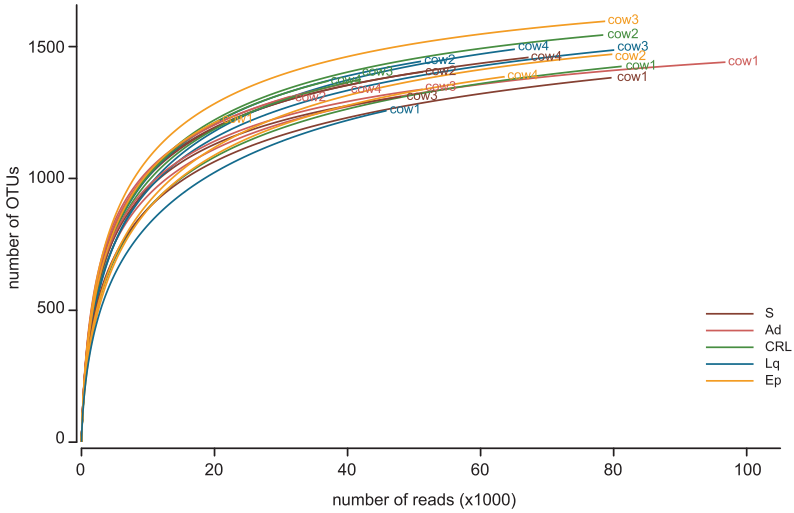


Figure S2.1 Rarefaction curves of the bacterial communities of five samples types collected from four cannulated cows. The sample types are: crude solids (S), the eluted solid adherent fraction (Ad), free-living species in the crude rumen liquid (CRL), strained liquid samples (Lq), and epimural scrapings (Ep).

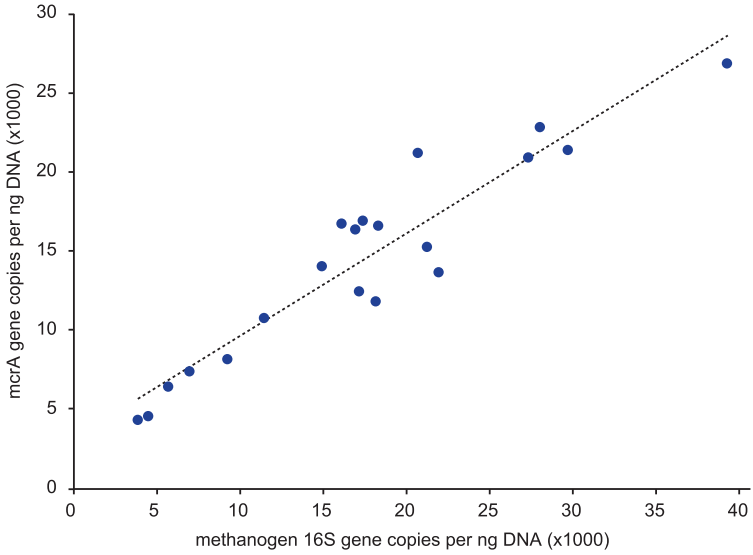


Figure S2.2 Scatterplot of the absolute concentrations of total methanogens, expressed as gene copies per ng DNA, measured with methanogen specific 16S primers or mcrA primers. The correlation is indicated by the linear regression line and the squared Pearson correlation coefficient was calculated ($R^2 = 0.8847$).

Chapter 3

Impact of breed on the rumen microbial community composition and methane emissions of Holstein Friesian and Belgian Blue heifers.

The content of this chapter is based on:

T. De Mulder, N. Peiren, L. Vandaele, T. Ruttink, S. De Campeneere, T. Van de Wiele, K. Goossens (2017) Impact of breed on the rumen microbial community compositions and methane emission of Holstein Friesian and Belgian Blue heifers, Livestock Science. doi: https://doi.org/10.1016/j.livsci.2017.11.009"

CHAPTER 3 IMPACT OF BREED ON THE RUMEN MICROBIAL COMMUNITY COMPOSITION AND METHANE EMISSSIONS OF HOLSTEIN FRIESIAN AND BELGIAN BLUE HEIFERS

Abstract

Intensive dairy and beef cattle farming significantly contribute to the emissions of greenhouse gases from Belgian agriculture. Two main breeds dominate the Belgian cattle livestock; Holstein-Friesian (HF) dairy cattle and double-muscled Belgian Blue (DMBB) beef cattle. The aim of our study was to quantify and compare methane emissions of both breeds under conditions of equal diet composition, environment and physiological stage (using heifers of the same age). The methanogen and bacterial communities were thoroughly investigated using metabarcoding to correlate taxonomic compositions with breed and methane emission levels. HF heifers had significantly higher absolute enteric methane emissions as compared to DMBB heifers. Methane production was positively correlated to the dry matter intake (DMI). Due to the significantly higher DMI and energy intake of HF heifers, methane yield per DMI was not significantly different between breeds. Furthermore, no significant differences were observed between the gross feed efficiency (GFE) of both breeds, but the DMBB heifers demonstrated significantly lower CH_4:CO_2 ratios (mole-to-mole ratio), suggesting a more efficient carbon conversion of the feed. Although both breeds accommodated a common core of taxonomic groups, the bacterial communities also showed a breed specific composition due to differential abundance of specific species belonging to the main taxonomic groups and the presence of a few species of minor taxonomic groups that were significantly associated with one of both breeds. In contrast to the bacterial communities, the methanogen community was consistent and stable between breeds and at different sampling times. Our results suggest that breed related factors influence the bacterial community composition, while the variation in methane emission levels can be attributed mainly to the feed intake of the animals.

3.1 INTRODUCTION

The impact of intensive agriculture on climate change can for a large part be attributed to the production of greenhouse gases (GHG) by livestock breeding. GHG emissions from agricultural practices accounted for 8.5% of the total Belgian emissions in 2014. 45% of these emissions could be attributed to methane production during enteric fermentation by cattle [299]. In 2015, Belgium counted around 2.5 million cattle and an almost fifty-fifty distribution of dairy to beef cattle [300]. The majority of dairy cattle are from the Holstein-Friesian (HF) breed while beef cattle in Belgium are mainly Double-Muscled Belgian Blue (DMBB) [301]. Decades of intensive breeding and trait-selection have ensured that both breeds are respectively optimized for the production of either milk or meat yield and have resulted in distinct genotypical and phenotypical differences between HF and DMBB. DMBB cattle are known for its exceptional musculature as a results of the heritable inactivation of the myostatine gene [302]. The myostatine mutation is pleiotropic in its effect and also influence the internal organ sizes of DMBB, which are smaller than in most other breeds. Consequences of a smaller digestive tract are the reduced feed intake capacity and the improved feed efficiency [302] which results in distinct nutritional requirements for DMBB [303]. Previous studies have determined that the bacterial community composition and methane production is strongly influenced by genetic variation of the host animals [115,151]. Furthermore, many studies on both dairy and beef cattle have reported strong correlations between methane productions and live weight, dry matter intake (DMI) and gross energy intake (GEI) [304–307]. It is, however, unknown whether the production-related physiological and morphometric characteristics of DMBB cattle have an impact on their methane production and their rumen microbial community composition. Despite the importance of the DMBB breed for the Belgian meat production, methane emission data of DMBB cattle is scarce [308,309] and to our knowledge, the microbial community in the rumen of DMBB has not yet been investigated. The aim of this study was therefore to evaluate the differences in the rumen microbiome and in the enteric methane production between the HF breed and the DMBB breed under conditions of equal diet, housing and physiology stage.

3.2 MATERIAL AND METHODS

3.2.1 Animals, diet, methane measurement and rumen sampling

Eight HF and eight DMBB heifers of similar age (averaging 23.3 ± 1.5 months) and gestation stage (averaging 6.5 ± 1.9 months) were co-housed in a free stall. At the start of the experiment, the HF and DMBB heifers had an average weight of 558 ± 39 kg and 594 ± 42 kg, respectively. Throughout the experiment, all heifers were fed the same diet to minimize dietary influences on the rumen microbial community and the methane production of the animals. The basal diet consisted of 40% maize silage, 40% grass silage and 20% grass hay (on dry matter basis) and was complemented with concentrate feed (Table 3.1). Cows had *ad libitum* access to roughage in Roughage Intake Control (RIC, Insentec, Marknesse, The Netherlands) feeding bins and concentrate was provided by the GreenFeed system (C-Lock Inc., Rapid City, USA) with a daily maximum of 1.3 kg. Daily roughage and concentrate intake was individually monitored and live weight was measured biweekly. During a two week period, the heifers adapted to the diet and were trained in eating from the RIC bins and visiting GreenFeed. Following this adaptation period, enteric CH_4 and CO_2 emissions (g d^{-1}) were measured over a period of six consecutive weeks using the GreenFeed system [310]. On average, the heifers visited the GreenFeed system five times per day without significant differences between breeds. The least visiting animal had an average of three visits per day during the measurement period. Methane emissions per cow (g d^{-1}) were calculated as the average over all measurements (no animals had to be excluded due to insufficient number of data points). The rumen degradable protein balance (RDPB) was calculated as described by Tamminga et al. (1994) [311]. Crude fat, crude protein and starch content, neutral detergent fiber (NDF), net energy (NE) and protein digested in the intestine (DPI) were determined like in De Boever et al. (2017) [312].

Rumen fluid samples were collected using a FLORA rumen scoop (Products of Professor Geishauser, Wittebreut, Germany) on two sampling days, on day 36 and on day 42 during the six-week measurement period. Samples were kept in sterile falcon tubes on ice during transport to the lab and aliquots of 1 ml were stored in cryovials at -80°C prior to DNA extraction. One DMBB heifer calved at the end of the experimental period but before the last rumen sample collection. Therefore this cow was excluded from the amplicon sequencing analysis.

The experimental setup (sample collection) and animal housing conditions were evaluated and approved by the Animal Ethics Committee of ILVO (reference EC2015/252).

Table 3.1 Diet composition

	g kg^{-1} dry matter
Crude fat	22.8
NDF	434
Starch	149
Crude protein	148
NE (MJ/kg)	5.7
DPI	69
RDPB	23

3.2.2 DNA extraction

DNA extractions were carried out exactly as described in Chapter 2, section 2.2.2.

3.2.3 16S rRNA amplicon sequencing and data processing

Metabarcoding of bacteria and methanogen communities was done on rumen fluid samples collected from 8 HF and 7 DMBB heifers on two sampling days (n = 30) on an Illumina MiSeq PE300 (Macrogen). Preparation of the amplicons and processing of the sequenced reads was carried out as described in Chapter 2, section 2.2.3. The raw sequenced data is stored in the NCBI short Read Archive (SRA), accession number ID SRP111912. The processing procedure resulted in an average of 99 971 reads per sample, with an average read length of 417 bp for the bacterial dataset and an average of 10 181 reads per sample, with an average read length of 451 bp for the methanogen dataset. Using QIIME, rarefaction curves were calculated using an upper rarefaction depth of 30 000 sequences, to ascertain if the sequencing depth was sufficient to measure the true alpha diversity (data not shown). The final OTU table was normalized using cumulative sum scaling (CSS) [313] to account for variable library sizes, using the QIIME script “normalize_table.py”.

3.2.4 Downstream data analysis

Shannon-Wiener diversity, Simpson diversity indices and observed richness were calculated with the Phyloseq package in R [264]. For subsequent data analysis, only OTUs representing at least 0.01% of the total bacterial community in at least one sample were retained thus reducing the total number of OTUs from 3083 to 2521. Multivariate analysis of the datasets was done using the R package Vegan. The betadisper function was used to ascertain the multivariate spread of the data. If multivariate homogeneity of group dispersions was fulfilled, differences between communities from both breeds and sampling times were analyzed with

PERMANOVA analysis, using the adonis function. Bacterial and methanogen community similarity between rumen samples from the DMBB and HF heifers were visualized with non-metric multidimensional scaling (NMDS) of the Bray-Curtis dissimilarity matrix, using the isoMDS function [263]. In case of significant PERMANOVA results ($P < 0.05$) and separate clustering in NMDS a generalized linear model (GLM) analysis, using the glm pipeline as implemented in the R-package EdgeR [314], was used to identify differentially abundant OTUs that contribute significantly to the community differences between the two breeds and the two time points. The core members of microbiomes in the rumen of HF and DMBB were identified using Corbata [315]. OTUs were regarded as core member if they had a relative abundance of at least 0.01% (0.1% for methanogens) in at least 80% of the samples. For the methanogen dataset, an OTU level heatmap was generated using the R-package gplots and the heatmap.2 function, using Manhattan distances and UPGMA (unweighted pair group method with arithmetic mean) for hierarchical clustering of samples.

3.2.5 Quantitative PCR

QPCR analysis to quantify total bacteria and total methanogens were performed using the primers, equipment and PCR conditions as in Chapter 2, section 2.2.5.

3.2.6 Statistical analysis

Statistical analysis with the two-sample t-test was used to determine significant differences between HF heifers and DMBB heifers in terms of methane production, DMI, gross feed efficiency and growth. Beforehand, the assumptions of normal distribution and homoscedasticity were verified with the Shapiro-Wilk test and the Fisher's F-test, respectively. The Benjamini-Hochberg procedure was used to adjust for multiple testing. The Pearson correlation coefficient between methane emissions and physiological and nutritional metrics were calculated. All statistical analysis were conducted in R (R version 3.2.2).

3.3 RESULTS

3.3.1 Feed intake, growth and methane emissions

The HF heifers had a significantly higher intake of roughage, concentrate, protein and net energy (NE) as compared to the DMBB heifers (Table 3.2). Coinciding with the higher feed intake, the HF heifers also had a significantly higher daily weight gain (DWG) ($p < 0.05$) of

1205 ± 184 g d^{-1}, whereas DMBB heifers gained on average 917 ± 187 g d^{-1} during the experiment. The gross feed efficiency (GFE), calculated as the ratio of DWG to DMI, was comparable between both groups (Table 3.2).

Table 2 The daily intake expressed in dry matter, protein and energy values (mean ± standard deviation), the gross feed efficiency (GFE) and the methane emissions of HF and DMBB heifers expressed per day, per dry matter intake (DMI), per daily weight gain (DWG) and as ratio to CO_2.

	DMBB	HF	P-value
DMI (kg d^{-1})	8.10 ± 0.67	10.43 ± 0.77	< 0.001
roughage	7.35 ± 0.66	9.57 ± 0.79	< 0.001
concentrate	0.74 ± 0.11	0.86 ± 0.04	< 0.05
DPI	565 ± 43	716 ± 43	< 0.001
RDPB	46.0 ± 3.7	59.2 ± 4.3	< 0.001
NE (MJ d^{-1})	46.0 ± 3.7	59.2 ± 4.3	< 0.001
GFE (kg kg^{-1})	0.115 ± 0.029	0.116 ± 0.017	p = 0.46
CH_4 (g d^{-1})	223 ± 16	264 ± 12	< 0.001
CH_4 per DMI (g kg^{-1})	27.6 ± 2.4	25.5 ± 2.1	p = 0.05
CH_4 per DWG (g kg^{-1})	253 ± 53	223 ± 31	p = 0.10
CH_4:CO_2 (moles:moles)	0.080 ± 0.006	0.091 ± 0.003	< 0.001

HF heifers produced significantly more methane than DMBB heifers. After correcting for DMI, the methane yield tended to be higher for DMBB than for HF heifers. Numerically, HF heifers had an 8% lower methane yield (CH_4 per DMI) as compared to DMBB heifers. Alternatively, methane production can be expressed in relation to the DWG, which was not significantly different between both breeds. The ratio between the emitted CH_4 and CO_2 is a measure of the feed carbon conversion efficiency for a given diet [316], reflecting the breed differences in rumen fermentation (i.e. microbial CH_4 and CO_2 production) as well as intermediary metabolism processes of the animal (body maintenance, fat/muscle deposition, etc.) that contribute to cellular respiration and CO_2 production. The CH_4:CO_2 ratio was significantly higher for HF heifers as compared to DMBB heifers (Table 3.2). Pearson correlation analysis indicated a positive relation ($r > 0.60$) between absolute CH_4 emissions and DMI ($r = 0.80$) and absolute CH_4 and absolute CO_2 emissions ($r = 0.63$) (Figure S3.1). No correlation was found between the gross feed efficiency and the absolute methane emissions ($r = 0.002$).

3.3.2 Bacterial community composition

The average rumen bacterial richness and diversity did not significantly differ between HF heifers (richness: 2353 ± 142 OTUs; Shannon diversity: 6.440 ± 0.097; Simpson diversity: 0.996 ± 0.001) and DMBB heifers (richness: 2352 ± 126 OTUs; Shannon diversity: 6.446 ± 0.090; Simpson diversity: 0.996 ± 0.001) and neither did the rumen bacterial densities (16S gene copies ng^{-1} DNA) (Figure 3.1).

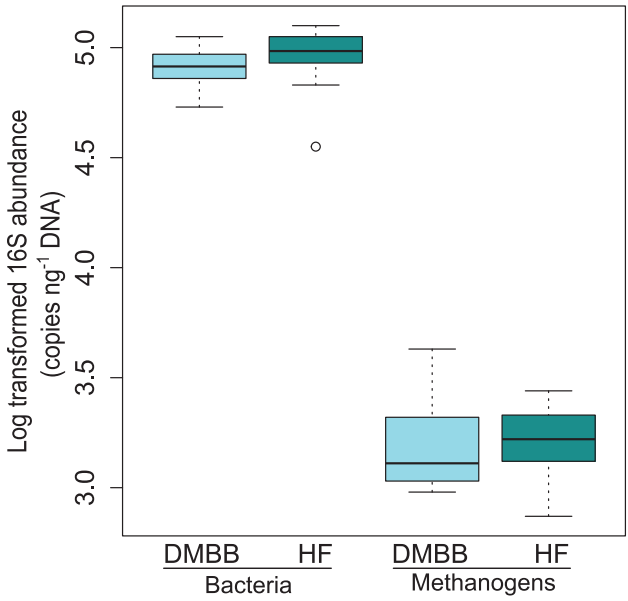


Figure 3.1 Box plot of 16S gene abundances of total bacteria and total methanogens in the rumen of DMBB (**light**) and HF (**dark**) heifers, as expressed by 16S gene copies per ng DNA yield.

The core microbiome across the HF and DMBB samples consisted of 897 OTUs, of which 137 OTUs had a relative abundance above 0.1% in at least 80% of the samples. The abundant core members were represented primarily by OTUs from the *Prevotellaceae* (37 OTUs), *Ruminococcaceae* (25 OTUs), *Lachnospiraceae* (16 OTUs), *Rikenellaceae* (RC9 Gut Group) (14 OTUs), BS11 Gut Group (7 OTUs) and the *Fibrobacteraceae* (5 OTUs) and 33 OTUs belonging to other minor taxonomic groups or unassigned. Cumulatively these core members represented 35.6% of the OTUs and comprised 83.1 ± 2.4% of the reads.

NMDS maps observed community dissimilarities (as calculated by the Bray-Curtis principle) non-linearly onto an ordination space and thus visualizes the differences between the

bacterial community compositions in the rumen of both breeds. The bacterial communities of the DMBB heifers cluster separately from the HF heifers, but also samples collected from the same heifers but on two different time points (one week apart) seem to cluster separately (Figure 3.2). Significant differences in Bray-Curtis similarity indices (permutation based PERMANOVA) confirmed the separation of samples according to breed ($p = 0.001$) and sampling time ($p = 0.002$), suggesting an influence of both factors on the bacterial community composition. Notably, the intra-breed rumen bacterial community composition is more similar for HF heifers than DMBB heifers, as the DMBB samples are spread more in the NMDS. Differential abundance analysis identified 51 OTUs as significantly differentially abundant between the two sampling times and 283 OTUs as significantly differentially abundant between both breeds, of which 124 OTUs were significantly less abundant and 159 OTUs were significantly more abundant in the rumen of HF heifers as compared to DMBB heifers. In other words, 11.2% of the OTUs and 13.2% of the reads contribute to the separate clustering according to breed on NMDS. To verify if these identified OTUs are indeed responsible for the observed differences between the bacterial communities of HF and DMBB, NMDS analysis was performed on the dataset excluding the differentially abundant OTUs as well as a dataset consisting solely of differentially abundant OTUs (Figure S3.2). The significances ($-\log_{10}P$) and the log2-fold changes of the differentially abundant OTUs are visualized in a volcano plot (Figure S3.3). Differentially abundant OTUs were selected with a fold change smaller than -1 or larger than 1 and a P-value below 0.01. These differentially abundant OTUs belong to 38 families. Abundant families (Figure S3.4) as the Prevotellaceae, Lachnospiraceae, Ruminococcaceae, Spirochaetaceae (represented mainly by *Treponema* sp.), BS11 gut group and S24-7 (Bacteroidales) and the RC9 gut groups (Rikenellaceae) contained OTUs with differential abundance in both breeds. OTUs belonging to Coriobacteriaceae, *Porphyromonas*, *Bergeyella*, Candidate division TM7, *Thalassospira*, *Mannheimia* and *Acinetobacter* had higher relative abundances in DMBB heifers, whereas OTUs of RF16 (Bacteroidales), *Fibrobacter*, vadinBB60 (Clostidiales), Veillonellaceae (represented by *Anaerovibrio* and *Selenomonas*), Lentisphaerae, Planctomycetaceae and Succinivibrionaceae had higher relative abundances in HF heifers.

3.3.3 Methanogen community composition

Similar to the bacterial population, the average methanogen richness and diversity did not statistically differ between DMBB heifers (richness: 17.0 ± 1.0 OTUs; Shannon diversity: 1.566

± 0.147; Simpson diversity: 0.700 ± 0.037) and HF heifers (richness: 16.7 ± 1.0 OTUs; Shannon diversity: 1.535 ± 0.107; Simpson diversity: 0.680 ± 0.032). A total of 19 OTUs were detected across all samples, belonging to three families: *Methanobacteriaceae* (89.1 ± 4.0% of the reads), *Methanomassiliicoccaceae* (10.8 ± 4.0%) and *Methanosarcinaceae* (0.04 ± 0.04%). The hierarchically clustered heatmap of the methanogen communities in the rumen of HF and DMBB, does not indicate separate clustering of samples according to breed or sample time. Instead the communities consisted of a core of 11 OTUs and were distinct only in terms of (small) differences in the relative abundances of the OTUs, without any pattern (Figure 3.3). The total methanogen community made up 1.95 ± 0.66% of the prokaryotic community (ratio of 16S gene abundances) and the absolute abundances of methanogens (16S gene copies ng^{-1} DNA) were not different between breeds (Figure 3.1).

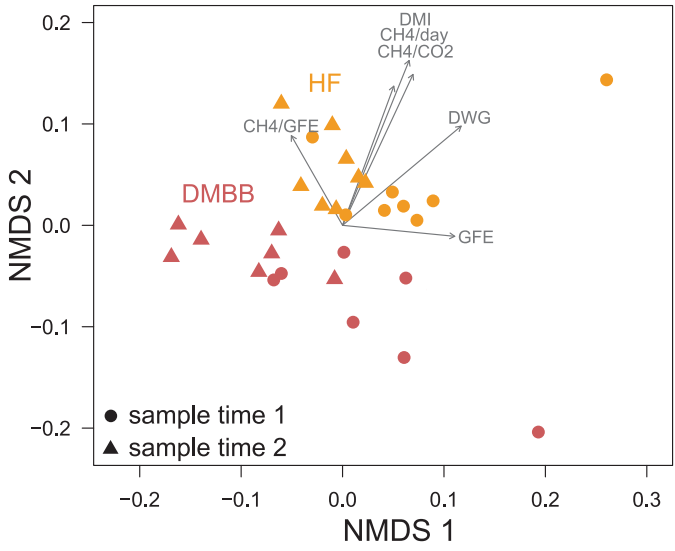


Figure 3.2 NMDS ordination of pairwise community dissimilarity (Bray-Curtis) indices of bacterial 16S sequencing data from rumen samples of DMBB and HF heifers on d36 and d42 of the 6-weeks measurement period. Arrows indicate significant correlation ($p < 0.05$) of the ordination with nutritional or fermentative parameters.

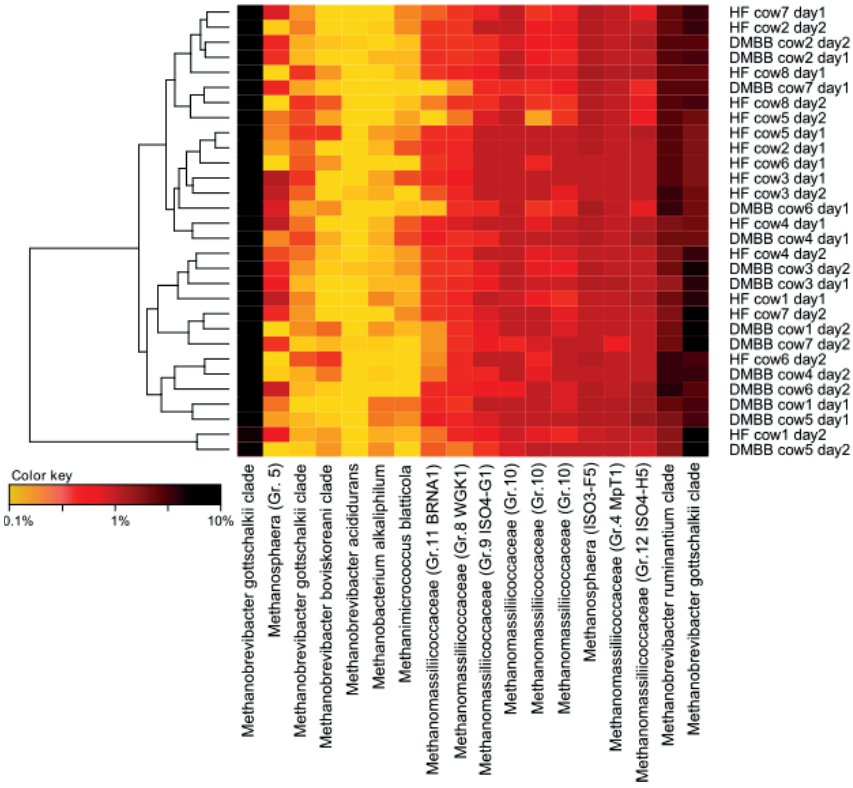


Figure 3.3 Heatmap of the methanogen OTUs, identified until the lowest available taxonomic level (**horizontal**) from rumen samples of DMBB and HF heifers on two different time points (**vertical**). The dendrogram indicates the community resemblance based on UPGMA clustering and the Manhattan distance method

.

3.4 DISCUSSION

Heifers were selected based on age and gestation length, co-housed in the same cattle pen and provided the same diet so to limit the influence of physiological stage, ambient conditions and feed composition on the rumen microbial ecosystem. The major variable left between HF and DMBB heifers was the difference in DMI intake: DMBB heifers had a 22% lower DMI than HF heifers, in line with expectations based on previous reports [317,318]. As a consequence of the lower dry matter and energy intake of DMBB heifers, these heifers produced significantly less methane (g d^{-1}) as compared to HF heifers. However, significant differences in methane emissions were not observed when CH_4 production was corrected for DMI. The lower DMI implies that rumen bacteria of DMBB are provided with less substrate for enteric fermentation,

resulting in a lower availability of H_2 for methanogenesis. Despite their lower DMI, DMBB cattle are known for their better feed conversion efficiency compared to other cattle breeds. However, in our study no significant differences were found between gross feed efficiency (GFE) of HF and DMBB Heifers. This is likely a consequence of the experimental design with equal dietary conditions for both breeds. The diet used in this trial was formulated to meet the nutritional requirements of pregnant HF heifers in order to calve in optimal body condition and to avoid metabolic problems after calving. As DMBB heifers have a lower intake capacity, an energetically denser diet is usually provided, especially at the end of gestation. Despite the suboptimal dietary conditions for DMBB, the heifers realized an average daily growth of 917 g per day, higher than the recommended growth rate of 600 - 790 g per day for pregnant DMBB heifers of this age [319]. Furthermore, the DMBB heifers in this trial had a significantly lower CH_4:CO_2 ratio as compared to HF, indicating a more efficient carbon conversion of the feed by DMBB [316,320]. A positive correlation was found between DMI and absolute methane emissions and absolute methane and CO_2 emissions. Our conclusions are in line with the results of Rooke et al. (2014) who assessed how various dietary conditions, cattle genotype and the rumen microbiome affect H_2 and CH_4 emissions in beef cattle breeds. The Aberdeen Angus-sired steers had a higher DMI and produced more CH_4 than Limousin-sired steers, but these differences were not observed when CH_4 emissions were expressed per DMI or per GEI [145].

The CH_4 yield of DMBB measured in our study (27.6 g kg^{-1} DMI) is higher compared to those reported by Castro-Montoya et al. (2015) [308] (17.1 g kg^{-1}). The observed differences in CH_4 yield can be attributed to the dietary compositions. Castro-Montoya and colleagues provided a diet consisting of maize silage and concentrate in a 50:50 ratio on DM basis, with a lower fiber content (NDF: 307 g kg^{-1} versus 434 g kg^{-1} in this experiment) and a higher starch content (208 g kg^{-1} versus 149 g kg^{-1}) as compared to the diet in the current study. However, the dietary effect is likely magnified by the different method of methane measurement (GreenFeed system versus respiratory chamber) and possible differences in animal physiology. Nevertheless, this comparison emphasizes that DMBB heifers would have a significantly lower methane yield if they were fed a diet optimized for their own energy requirements.

Our experimental setup, in which we tried to minimize dietary and physiological influences, allowed to study the influence of the breed on the rumen bacterial and methanogen community composition. The rumen of both breeds accommodated similar methanogen densities and a common core of methanogen OTUs, belonging to only a few taxonomic groups.

These observations are in accordance with Cersosimo et al. (2016), who concluded that Holstein and Jersey cows carry the same core methanogen community. Their results indicated an influence of lactation stage and diet on the methanogen community composition, but no difference between breeds was observed for the same lactation stage and under identical dietary conditions [322]. Moreover, the methanogen community in the foregut proved to be highly conserved among 32 different (sub-) species of ruminants from seven global regions [103]. Within the rumen ecosystem, the methanogen population performs a specific terminal role in the electron transfer chain driven by anaerobic fermentation. Their high affinity for H_2 enables methanogens to maintain a stable community despite their low richness and diversity. As reviewed by Tapio et al. (2017) [323], most studies have found no correlation between the overall methanogen abundance and methane emissions. The methanogen's gene expression, rather than gene abundance might be a better proxy for methane emission.

In contrast to the methanogen community, NMDS analysis of the bacterial community composition showed a separate clustering of samples according to breed, pointing either to a breed specific rumen microbiome composition or an influence of the life trajectory before the start of the trial. Evidently, breed related factors can exert an influence on the bacterial community composition by benefiting specific species. These factors include the phenotypical, physiological and possibly the immunological differences between breeds but presumably, also early life trajectory such as the conditions of birth (DMBB calves were born by C-section), breed specific housing, rearing strategies and diet compositions in early life may play a role in the formation of a breed-specific rumen microbiome. In agreement with the findings of Henderson et al. (2015) [103], a core microbiome of dominant rumen bacteria was similar for all samples of both breeds, composed primarily of species of the Prevotellaceae (mainly *Prevotella*), Rikenellaceae (RC9 gut group), BS11 gut group, Ruminococcaceae (including *Ruminococcus*) and Lachnospiraceae (including *Blautia*, *Butyrivibrio*, *Coprococcus*) Fibrobacteriaceae (mainly *Fibrobacter*), Acidaminococcaceae (mainly *Succiniclasticum*) and Succinivibrionaceae. Tapio et al. (2017) recently reviewed the literature on associations between the bacterial rumen composition and methane emission and found that some species within the Prevotella and other larger taxonomic groups are correlated with high methane production while others are more dominant in the rumen of low methane emitters. The taxa that are associated with high or low methane emitters (according to [323]) did not have differential abundance in HF or DMBB. However, within the most dominant families, shifts in species abundance were observed between both breeds. The families Prevotellaceae, Ruminococcaceae

and Lachnospiraceae are the most dominant constituents of the rumen bacterial community in both breeds and included common OTUs as well as OTUs that were differentially abundant in both breeds. The Prevotellaceae is mainly represented by the genus of *Prevotella* which includes members with proteolytic, amylolytic and hemicellulolytic activity [324], while the Ruminococcaceae and Lachnospiraceae are known to contain hemicellulolytic and cellulolytic species [280–282]. The functional versatility of species in these families could be the reason that some species are abundant in the rumen of HF heifers whereas others are more abundant in the rumen of DMBB heifers. Furthermore, we observed a remarkable shift within the Proteobacteria of HF and DMBB: Succinivibrionaceae were about twice more abundant in HF than DMBB (-1.15 $\log_2$FC), whereas the Pasteurellaceae, Moraxellaceae, Desulfuromonadales and the Rhodospirillaceae are significantly more abundant in DMBB. Deeper taxonomical identification of these OTUs indicated the presence of *Mannheimia haemolytica*, a commensal of the nasopharynx and an opportunistic pathogen causing bovine respiratory disease [325,326], *Acinetobacter lwoffii*, a normal bacterium in the oropharynx but associated with a number of infectious diseases in humans including pneumonia and gastroenteritis [327], and *Escherichia coli*, a known commensal of the intestinal tract but with pathogenic serotypes. The increased abundance of these potential pathogens in the rumen of DMBB might be associated with the frequent health problems observed in DMBB calves. DMBB calves often suffer from cardiorespiratory diseases which could be attributed to the pleiotropic effect of myostatine mutations, the smaller lung volume and pure line breeding [328]. In accordance with human studies reporting an association between the way of delivery (vaginal delivery versus C-section), the gastro-intestinal microbiome, and later health problems [329–331], we hypothesize that DMBB calves, routinely born by C-section, might have a breed-specific predisposition for certain diseases. Although the opportunistic pathogens found in the rumen of DMBB heifers are likely not active members of the rumen microbial ecosystem but end up in rumen with the saliva inflow, more dedicated studies are needed to investigate a possible relationship between C-section, the rumen and intestinal microbiome composition and frequently reported health problems in DMBB calves.

3.5 CONCLUSION

No significant differences were observed between GFE of both breeds, but the DMBB heifers did demonstrate significantly lower CH_4:CO_2 ratios, suggesting a more efficient fermentation by the rumen microbial ecosystem. Although both breeds accommodated a common core of taxonomic groups, the bacterial communities showed a breed specific composition as specific species from the main taxa and a few species from minor taxon were significantly associated with the HF or DMBB breeds. In contrast, the methanogen communities were consistent and stable between breeds and at different sampling times. Our results suggest that breed related factors (including early life events) influence the bacterial community composition, while the variation in methane emission levels can be attributed mainly to the feed intake of the animals.

3.6 SUPPLEMENTARY DATA

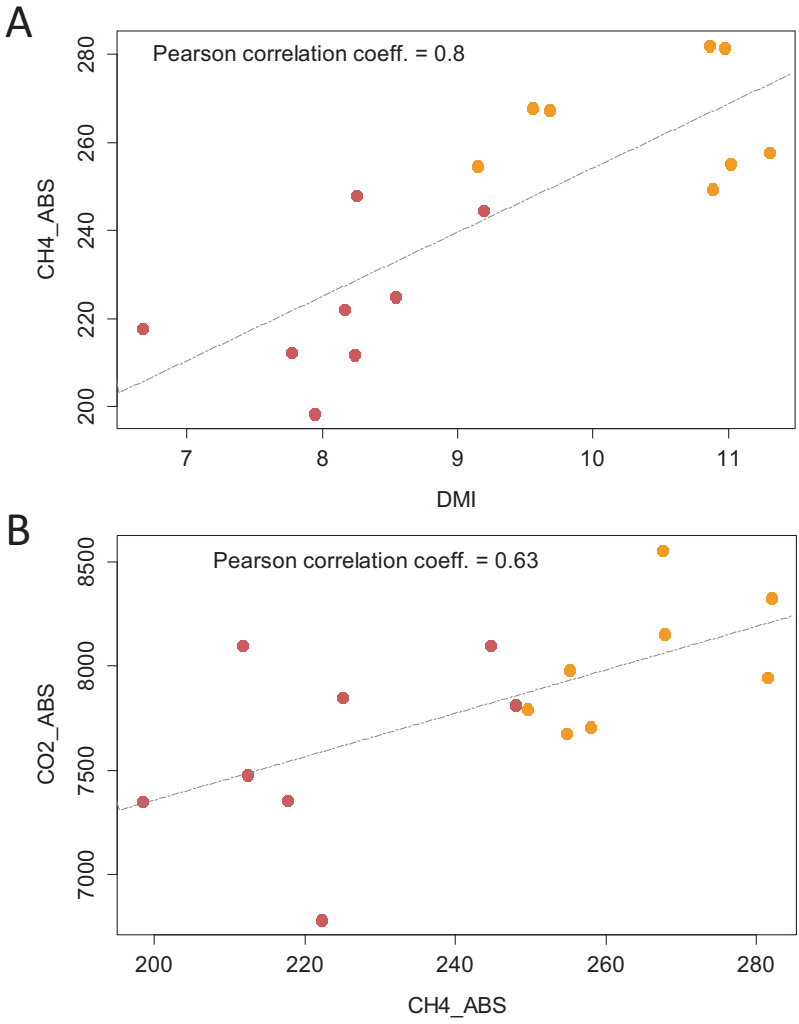


Figure S3.1 Scatter plots and regression line indicating the positive correlation between [**A**] the absolute methane emissions and DMI and [**B**] absolute CH_4 and CO_2 emissions. Yellow points indicate values from the HF heifers and red points from the DMBB heifers.

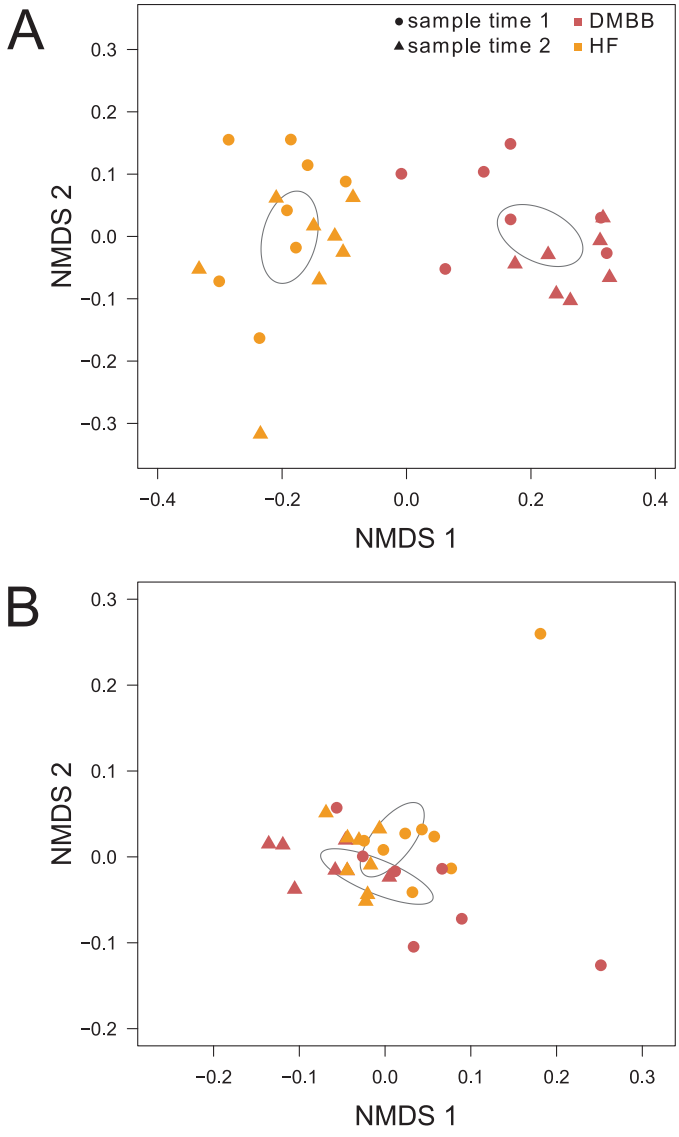


Figure S3.2 NMDS profile of pairwise community dissimilarity (Bray−Curtis) indices of eubacterial 16S sequencing data from rumen samples of DMBB and HF heifers on two different time points, **[A]** using a dataset of only the differential abundant OTUs (retaining 279 of 2521 OTUs) **[B]** and a dataset with the differential abundant OTUs removed (retaining 2242 of 2521 OTUs).

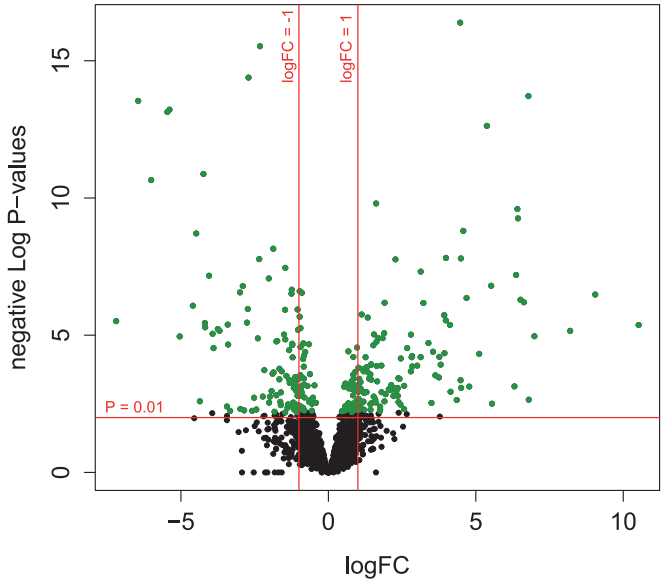


Figure S3.3 Volcano plot of the log2 fold change (logFC) and the negative log of the P-values (according to the differential abundance analysis with EdgeR) of all the OTUs in the dataset. The green points indicate those OTUs that were found to be differentially abundant between HF and DMBB.

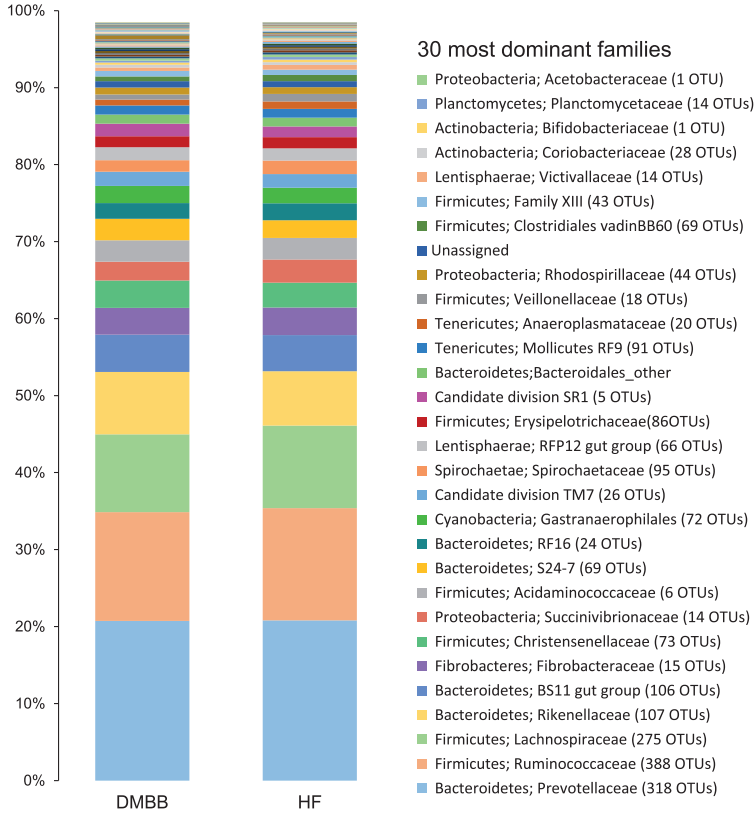


Figure S3.4 Bar chart of the average relative abundances of the bacterial families of the rumen bacterial communities of HF and DMBB.

Chapter 4

Host influences and dynamic fluctuations of the bacterial and methanogen community following a complete rumen content exchange

CHAPTER 4 HOST INFLUENCES AND DYNAMIC FLUCTUATIONS OF THE BACTERIAL AND METHANOGEN COMMUNITY FOLLOWING A COMPLETE RUMEN CONTENT EXCHANGE

Abstract

Understanding the rumen microbial ecosystem requires the identification of those factors influencing the microbial community composition. Diet composition is generally regarded as the driving factor affecting the microbial community composition, but also host related factors such as genotype, physiological state and life history are assumed to have an influence. The aim of this study was to evaluate the host effect on the rumen bacterial and methanogen communities following a rumen content transfer, under conditions of equal nutrition and physiological stage. Out of four cannulated Holstein Friesian cows (mid-lactation), one donor cow was selected based on its slightly higher methane production. The rumen content of the donor was thoroughly removed and used as inoculum for the emptied rumen of the donor itself and three acceptor cows. After the rumen content transfer, samples were collected at regular time-points to investigate the effects on volatile fatty acid (VFA) profiles, on the bacterial and methanogen community using metabarcoding and on the methane emissions. The response to the perturbation of the rumen ecosystem was different between cows. The donor and one of the acceptor cows had a brief depression in feed intake, methane emissions and altered VFA proportions. These short-term changes were reflected in the bacterial community the first two days after transfer: the richness decreased from 1500 to 800 OTUs and novel OTUs gained the opportunity to dominate the community. Following these circumstances, the rumen bacterial community underwent several autogenic successions in its search for a new steady state. The fermentation metrics of the two other acceptor cows were not affected as compared to the pretransfer values. Their rumen bacterial composition initially maintained the composition of the donor, but over time the bacterial community reached a new dynamic equilibrium that resembled neither the donor nor the original composition. The data suggests that the rumen bacterial community can restore quickly after a severe perturbation. In the absence of dietary influence, the composition is not solely host specific. Instead, the bacterial community is partly influenced by host-related factors but dynamic over time resulting in a well-balanced ecosystem with a core of stable and omnipresent species and transiently successive species. Opposite to

the bacteria, the methanogenic communities were unaffected by host effects and were stable over time.

4.1 INTRODUCTION

Nutrition is generally recognized as the primary factor influencing the rumen microbiome [332–334] although host related factors are also known to exert an influence on the microbial composition [103,151]. Diet alterations and feed additives are popular strategies to improve feed efficiency, to increase production or to lower methane emissions, but the effectiveness is often subject to between-animal variations [146,147]. Differences between enteric methane emissions of ruminants on the same diet and environmental conditions [103,115,116] also suggest an influence of host-related factors on the microbial activity and possibly the microbial composition. These host-related factors can be categorized into two general groups. (i.) The genotype related factors, which include those factors that could be influenced by host gene expression or genetic heritability. This group comprises for example the size of the rumen organ, which influences feed intake and passage rate; salivary excretion, which influences rumen pH; absorption of microbial end products by the rumen epithelium and host-microbial cross-talk genes. (ii.) The non-genotype related factors include the physiological state and the life trajectory (early life events as birth conditions, rearing strategy, weaning, previous diets and medical treatments).

The extent to which these host-related factors play a role in shaping the rumen microbial composition can be studied by a rumen content transfer between cannulated cows. Such an abrupt disturbance of the rumen microbial community allows investigating to what extent the host exerts an influence on the rumen fermentation and the new microbial community, while the latter strives for a stable ecosystem. The current study used a rumen content transfer from one donor cow to three acceptor cows and the donor cow itself. This setup created identical inoculating conditions, i.e. the microbial community of the donor was present in each of the four experimental cows. In the subsequent six weeks after transfer, the host specific influence on the formation of a stable new community was investigated by regular sample collection, with the primary focus on the dynamic methanogen and bacterial community composition and its relationship to fermentation metrics. This experiment aimed to determine if the methanogen and bacterial community would return to its original composition, thus mainly influenced by host related factors, if it would maintain the rumen microbial community composition of the donor or if it would evolve into a new microbial composition (Figure 4.1.A).

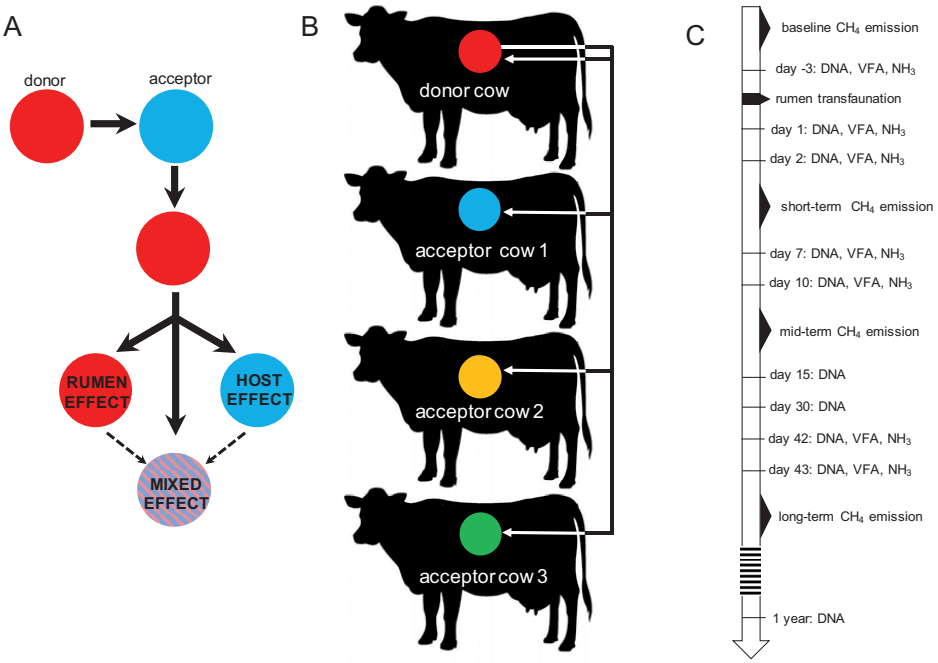


Figure 4.1 [A] Possible influences that could shape the rumen microbial community composition after a rumen content transfer. **[B]** Visualisation of the rumen transfer setup. Each colour represents a host specific rumen microbiome. **[C]** Chronological overview of the experiment, indicating methane measurement periods, sampling moments for volatile fatty acids (VFA) and ammonia (N-NH_3) quantification and DNA extraction.

4.2 MATERIAL AND METHODS

4.2.1 Animals, diets and rumen content transfer

Four rumen-cannulated Holstein Friesian dairy cows of similar weight and lactation stage were fed a diet with the same forage-to-concentrate ratio of 70:30 on dry matter basis. The forage was a mixture of prewilted grass and maize silage in the ratio of 58:42 on dry matter basis. The concentrates consisted of a balanced compound feed, rumen protected soybean meal and feed urea (69:30:1). After an adaptation period of six weeks, the rumen content of the highest methane emitting cow, i.e. the donor cow, was completely removed through the cannula, divided into four equal parts in sealed 25 litre CurTec drums and kept at constant temperature in a 38°C water bath. The rumen of three acceptor cows was completely emptied and the contents were weighted and discarded. After the rumen was emptied, the rumen wall was rinsed with sterile saline solution (9 g/l NaCl) to remove residual fibers and fluids as completely as

possible. Subsequently, one quarter of the donor's rumen content was introduced back in the emptied rumen of the donor cow itself and the three other parts were transferred to the rumen of the three acceptor cows (Figure 4.1.B). Animals were offered forage and water immediately after transfer and ten litre saline solution of 38°C was poured in the rumen through the cannula.

The transfer is regarded as timepoint zero. Samples were collected for volatile fatty acid (VFA) and ammonia-nitrogen ($N\text{-}NH_3$) quantification before transfer (day -3), on day 1, day 2, day 7, day 10, day 42 and day 43 after transfer. Samples for DNA extraction and subsequent qPCR and metabarcoding were collected simultaneously with the samples for VFA and NH_3 analysis, and additionally on day 15, day 30 and one year after the rumen content transfer (Figure 4.1.C). The use of cannulated animals was in accordance with the Belgian law for care of experimental animals (Royal Decision 14.05.2010) and the experimental setup (rumen content transfer and sample collection) was approved by the Animal Ethics Committee of ILVO (EC2014/224).

4.2.2 Methane measurements

Enteric methane (CH_4) and CO_2 emissions were measured by keeping the animals in individual gas exchange chambers [241] as described by Castro-Montoya et al. [308]. The before mentioned gas concentrations were measured in the exhaust gas from each chamber with an 8 min interval, using an INNOVA 1314 Photoacoustic Multi-gas Monitor (LumaSense Techn., Santa Clara, CA, USA). The CO_2 and CH_4 emissions of the experimental cows were measured during four measurement periods: before transfer (day -7 to -4), short term (day 2-5), mid-term (day 11-14) and long term (day 43-46) after transfer.

4.2.3 Sample collection

During each sampling day, six samples were collected on 7:45, 8:45, 10:00, 11:30, 14:00 and 17:00 to account for diurnal changes of VFA and NH_3. Animals were fed twice daily, immediately after sample collection at 7:45 and 17:00. Rumen fluid was collected through the cannula using a vacuum pump connected to a metal perforated sampling probe. To increase representativeness of the sample, the probe was replaced several times to collect fluid from different regions in the rumen. The rumen fluid (around 200 ml) was collected in an Erlenmeyer. The pH was measured with a mobile pH meter and the rumen fluid was subsequently supplemented with three drops of toluene to cease microbial activity. Ten ml of rumen fluid of each sampling time was pooled for VFA analysis and another ten ml was kept

for Kjeldahl analysis. The samples were stored at -20°C prior to analysis. During the sample collection at 8:45, an additional rumen fluid sample was collected for DNA extraction. In the lab, a 500 µl subsample was transferred to a cryovial and stored at -80°C.

4.2.4 Volatile fatty acids and ammonia-nitrogen measurements

The VFA measurements were performed out using a protocol derived from Getachew et al. (2001) [335] using an EC-1000 capillary column on a Varian® 3900 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) with the Compass software. N-NH_3 measurements were carried out with the manual titration method described by Voigt & Steger (1967) [336]. The laboratory procedures for processing and analysing rumen samples for VFA and N-NH_3 detection are accredited under the BELAC ISO17025 norm (ILVO-DIER-ANIMALAB; certificate number: BELAC T-315).

4.2.5 DNA extraction

DNA extractions were carried out as described in Chapter 2, section 2.2.2.

4.2.6 16S rDNA amplicon sequencing and data processing

Metabarcoding of bacteria and methanogen communities was done on samples collected on ten sampling days from collection time points per cow (n = 40) on an Illumina MiSeq PE300 (Macrogen). Preparation of the amplicons and processing of the sequenced reads were carried out as described in Chapter 2, section 2.2.3. The raw sequenced data is stored in the NCBI short Read Archive (SRA), accession number PRJNA378589. The processing procedure resulted in an average of 72 384 reads per sample, with an average read length of 418 bp for the bacterial dataset and an average of 22 219 reads per sample, with an average read length of 451 bp for the methanogen dataset.

4.2.7 Downstream amplicon sequencing analysis

Shannon-Wiener diversity, Simpson diversity indices and observed richness were calculated with the Phyloseq package in R [264]. For subsequent data analysis, only OTUs representing at least 0.1% of the total community in at least one sample were retained thus reducing the total number of OTUs from 2488 to 678. The betadisper function was used to ascertain the multivariate spread of the data. If homogeneity of the group dispersions was fulfilled, differences between communities from samples collected at different time points or from

different cows were analysed by PERMANOVA, using the adonis function in the R package Vegan [263]. A heatmap of the sample-wide abundances sorted OTU table was generated using the heatmap.2 function of the R package gplots. Samples are clustered with the unweighted pair-groups method using arithmetic averages (UPGMA) based on Bray-Curtis dissimilarities.

4.2.8 Quantitative PCR

QPCR analysis to quantify total bacteria and total methanogens were performed using the primers, equipment and PCR conditions as in Chapter 2, section 2.2.5.

4.3 RESULTS

4.3.1 Milk production and dry matter intake

Large variation was observed between the four cows in their response to the rumen content transfer at the level of feed intake and milk production. The donor cow and acceptor cow 3 suffered from a feed intake depression during the two days following the rumen content transfer, during which they displayed a preference for concentrate feed. In subsequent days, the feed intake restored for both cows although acceptor cow 3 had an average 20.6% decrease in feed intake over the next two weeks, as can be observed from the weekly averages of dry matter intake (DMI) (Figure S4.1). Daily milk production of each cow was prone to day-to-day differences and is therefore visualised by moving averages (per two days) to even out fluctuations (Figure S4.2). Prior to the rumen content transfer, the donor cow had the lowest average milk yield over a period of 10 days and showed a slightly increasing trend in the six weeks following the transfer. The milk yield of acceptor cow 1 was not negatively affected while the milk yields of acceptor cows 2 and 3 dropped immediately after transfer and followed a downward trend thereafter.

4.3.2 Fermentation metrics: methane and volatile fatty acids

Prior to the rumen content transfer, the baseline methane emissions were determined for the four cows. The donor cow had a 10.2% higher methane emission (g CH_4 per kg DMI) compared to the three other cows. Following the rumen content transfer, the methane emissions of the donor and acceptor cow 3 initially decreased (short term) and were in part restored at the mid and long term measurements, though both cows remained below their original levels. The rumen transfer did not affect methane emission levels of acceptor cows 1 and 2. However,

during the long-term measurement, acceptor cow 1 had a higher absolute emission whilst the feed intake remained unchanged, resulting in an increased methane production per unit of DMI (Figure 4.2.A). The feed conversion ratio (FCR) is an indication of the feed efficiency and is calculated by the ratio of feed consumption (DMI) to milk yield. Prior to the transfer, the donor cow had the highest FCR (i.e. the lowest feed efficiency) as compared to the other cows. Immediately after the transfer, the FCR had decreased for every cow but restored thereafter and even increased above the original values for the acceptor cows. Notably, during the long term measurements (6 weeks after the transfer), the acceptor cows all had a higher FCR than the donor (Figure 4.2.B).

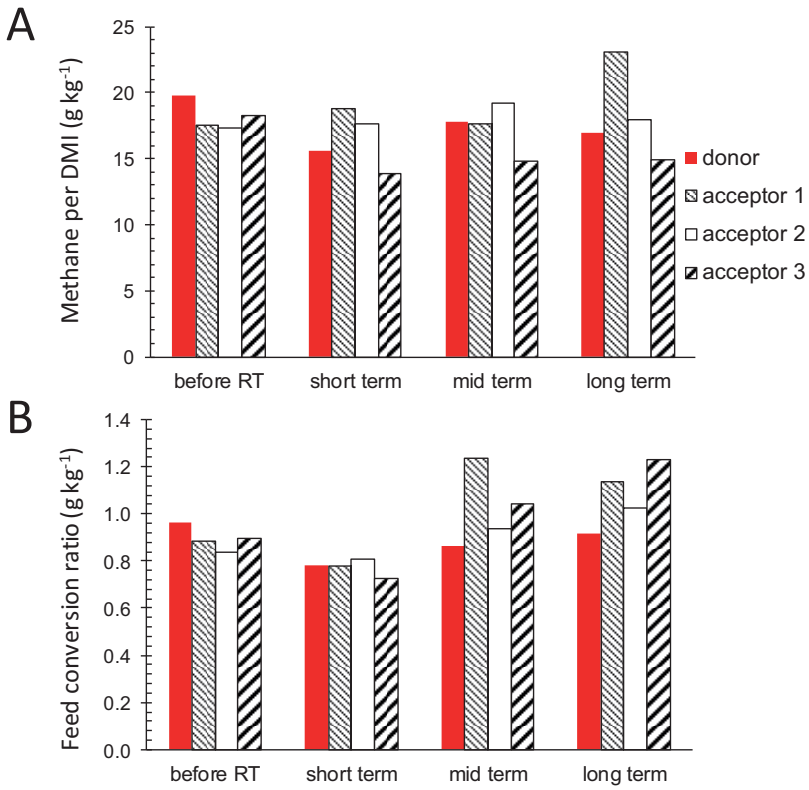


Figure 4.2 [A] Methane production per kg dry matter intake (DMI) and **[B]** the feed conversion ratio (or feed efficiency) calculated as the ratio of the DMI and the milk yield, at different times after the rumen transfer (short term = d2-5, mid-term = d11-14, long term = d43-46).

Generally observed, the cows had relative VFA proportions that approached a 60:20:20 ratio for acetate, propionate and butyrate. Directly following the rumen content transfer, the acetate portion decreased during a period of two days. The average decrease was more pronounced for the donor cow and acceptor cow 3 (-8.5%) as compared to acceptor cows 1 and 2 (-4.6%). Simultaneously, the propionate and butyrate portions slightly increased (+4.3% and +1.5) and the valerate portions more than doubled (+3.4%) for the donor cow and acceptor cow 3 while the increases were less for acceptor cows 1 and 2 (+2.0, +0.9% and +0.4% for Ac., Bu. And Val.). These alterations were temporary and in subsequent sampling days, the VFA proportions started to return to their original values (Figure 4.3).

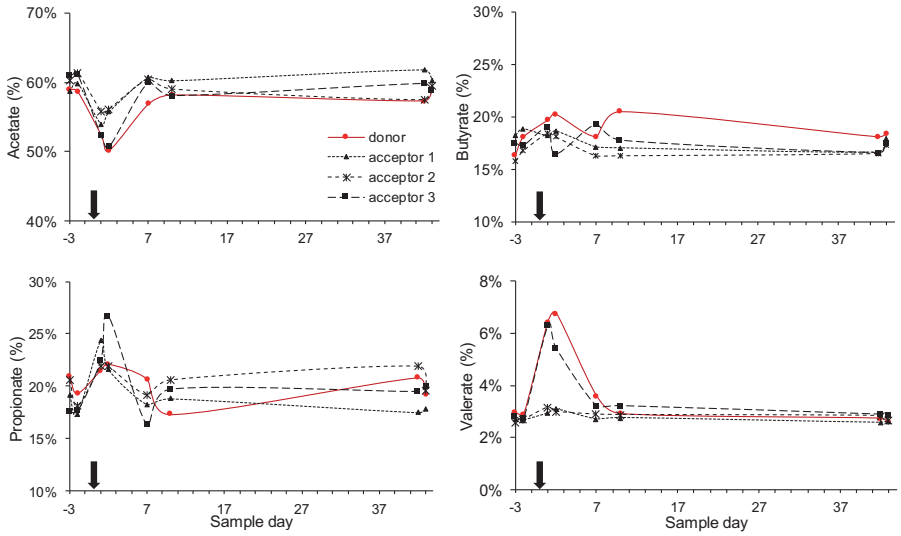


Figure 4.3 Percentage share of the main volatile fatty acids in the rumen fluid: acetate, butyrate, propionate and valerate in function of sampling time. The arrow indicate the moment of the rumen content transfer (i.e. day 0).

4.3.3 Bacterial and methanogen community

In the complete datasets, a total of 19 operational taxonomic units (OTU) were annotated as Methanoarchaea and 2393 OTUs as Bacteria. The bacterial richness in the rumen of the donor cow and acceptor cow 3 decreased from around 1500 to 800 OTUs in the two days following the transfer. Concomitantly also the evenness decreased as a few species gained dominance. Combined with the lower richness, this caused a major decrease of the Shannon and Simpson diversity measures (Figure 4.4.A).

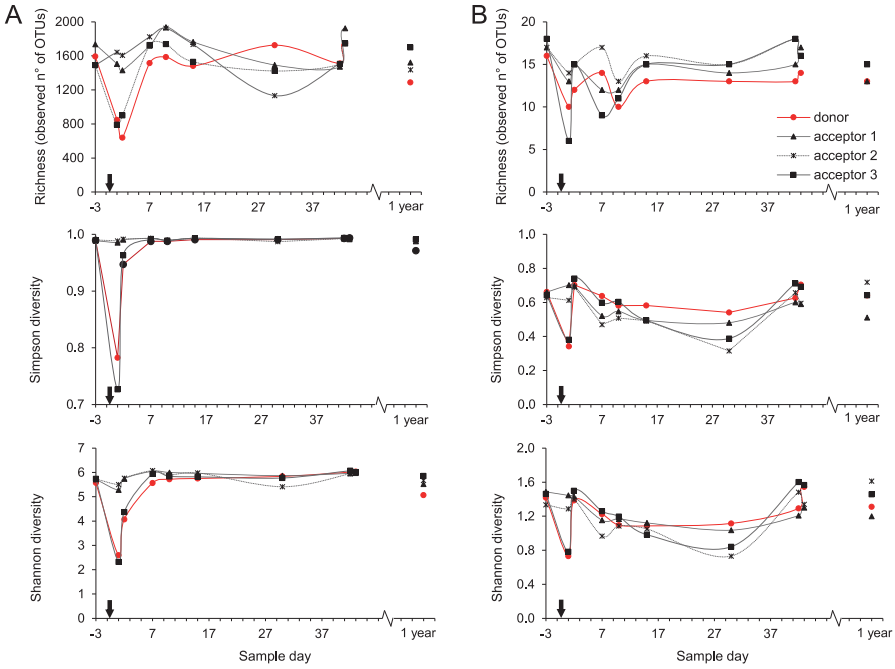


Figure 4.4 Richness, expressed as the observed number of operational taxonomic units (OTU), Simpson diversity and Shannon diversity of **[A]** bacterial and **[B]** methanogen populations at different time points. The arrow indicate the moment of the rumen content transfer (i.e. day 0).

Permutational multivariate analysis of variance (PERMANOVA) using the Bray-Curtis dissimilarities indicated significant differences between sampling days (not taking into account day 1 and day 2). The community profiles of the samples collected before the transfer were significantly dissimilar from the samples collected on all other sampling days ($p < 0.05$). The community profiles observed at day 7 and day 10 were significantly dissimilar from day 15 and day 30. The community profiles observed at day 15 were significantly dissimilar from day 30, day 42, day 43 and after 1 year, while day 30 only differs from 1 year ($p < 0.05$). These community differences are visualised using a heatmap with sample clustering according to Bray-Curtis dissimilarities (Figure 5A). Before the rumen content transfer, the bacterial profiles of the acceptor cows were similar. The first two days after the rumen content transfer, the bacterial community compositions of acceptor cows 1 and 2 showed striking similarities with that of the donor before transfer. The richness and diversity of the bacterial communities of the donor and acceptor cow 3 dropped immediately following the rumen content transfer, while about fifty novel OTUs gained temporary dominance. Noticeably, the enriched OTUs in these

samples were identified as *Prevotella bryantii* (OTU-1; 46.5%), *Sharpea azabuensis* (OTU-28; 5.6%), *Megasphaera elsdenii* (OTU-5; 10.6%), *Ruminococcus bromii* (OTU-20; 1.7%) and *Streptococcus bovis* (OTU-325; 0.7%) by individually matching the reference sequence for each OTU with the NCBI database (nBLAST) and RDP database (Sequence Match) (species levels are only reported in case of 100% sequence coverage and 100% identity on both databases) (Table 4.1). In the following week, the bacterial population strived to reach a new steady state and from day 10 and onwards, the bacterial profiles of all cows were determined by the sampling day and the host animal. The bacterial heatmap (Fig. 6A and Fig. S5) thus suggests a continuous and dynamic changing bacterial community with a core of ever-present OTUs with high relative abundances and groups of temporary abundant OTUs, which succeed each other in time. Moving window analysis of the community similarities of consecutive days (Figure S4.3) indicates that the community changes around 20 to 40% between each consecutive sampling time (Figure S4.4).

The Methanoarchaeal population was dominated by a mere four OTUs: two OTUs of the *Methanobrevibacter gottschalkii* clade (57.5 ± 12.3%) and *ruminantium* clade (19.5 ± 9.2%), one OTU of the *Methanosphaera* (5.7 ± 2.5%) and one OTU of the family of the *Methanomassiliicoccaceae* (7.7 ± 8.7%). During the brief feed intake deprivation of the donor cow and acceptor cow 3, decreases of the methanogen richness and diversity were observed during the first two days after transfer (Figure 4.4.B). In contrast, no influence was detected in the absolute quantity of bacteria and methanogens (Figure 4.6) or in the methanogen community profiles (Figure 4.5.B). The clustering of samples seems unaffected by the possible influences host, sampling time or “stress”. Only the sample of the donor cow collected one year after the ruminal content transfer differs from the other samples (Figure 4.5.B).

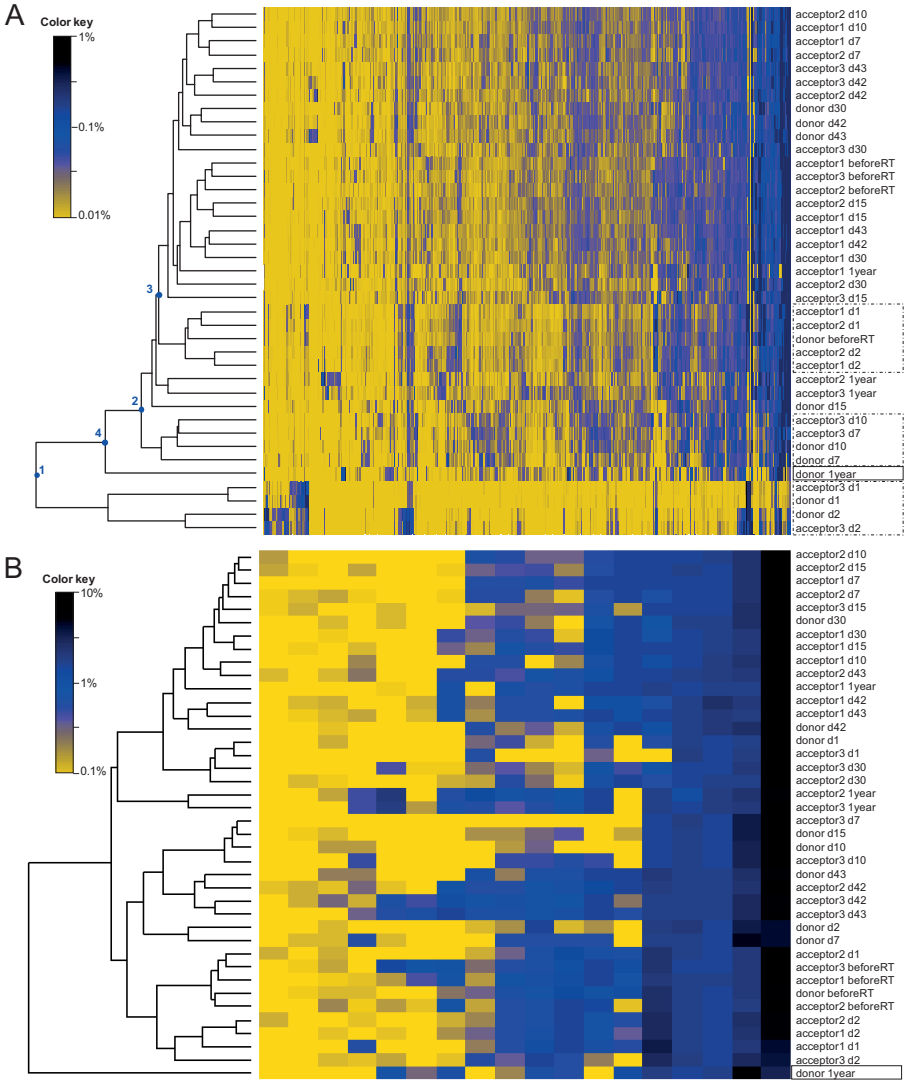


Figure 4.5 Heatmap of the **[A]** bacterial OTUs (vertical) and **[B]** methanogen OTUs in the rumen samples of four cows. The dendrogram indicates the community resemblance between samples based on UPGMA clustering and Bray-Curtis dissimilarity. After transfer, the donor cow and acceptor cow 3 suffered from feed intake deprivation which is reflected in the bacterial community profile, as they cluster separately (**node 1**). Also the samples collected on day 7 and 10 (**node 2**) of these cows cluster separately from the samples collected on later time points and other cows, suggesting that their rumen bacterial communities were still recovering. The samples collected from the acceptor cows before rumen transfer cluster together but separately from the donor sample before transfer. The first and second day after transfer, the bacterial profiles of acceptor cows 1 and 2 were similar to that of the donor cow before transfer (**node 3**). From day 7 and onwards, the bacterial community reached a new equilibrium and the position is seemingly determined by host and sampling time. Both the bacterial and methanogen community of the donor after 1 year, are dissimilar from all other profiles (**node 4**).

Table 4.1 List of the dominant OTUs detected in the samples one day after transfer in the cows suffering from physiological stress and feed intake deprivation (donor cow and acceptor cow 3)

OTU	Donor Day 1	Acceptor 3 Day 1	Donor Before	Average ± SD All samples*	Family	Genus
Core OTUs	Detected in every sample with high relative abundances					
OTU-1	43.6%	49.5%	1.47%	0.60 ± 0.73%	Prevotellaceae	*Prevotella*
OTU-8	0.13%	0.36%	1.84%	1.31 ± 0.76%	Prevotellaceae	*Prevotella*
OTU-7	9.81%	10.65%	0.39%	0.23 ± 0.20%	Prevotellaceae	
OTU-3	1.35%	1.40%	3.46%	4.42 ± 1.63%	Acidaminococcaceae	*Succiniclasticum*
OTU-48	0.15%	0.14%	0.88%	1.36 ± 0.52%	Christensenellaceae	
OTU-23	0.13%	0.14%	0.86%	1.04 ± 0.27%	Lachnospiraceae	Incertae Sedis
OTU-13	0.38%	0.60%	0.46%	0.47 ± 0.24%	Lachnospiraceae	*Pseudobutyrivibrio*
OTU-80	2.03%	1.39%	1.01%	0.44 ± 0.30%	Veillonellaceae	*Selenomonas*
Core OTUs	Not detected in every sample with high abundances but present in the donor sample before transfer					
OTU-6	4.13%	3.34%	2.02%	0.41 ± 0.58%	Bifidobacteriaceae	*Bifidobacterium*
OTU-39	0.11%	0.07%	0.33%	0.54 ± 0.22%	Acidaminococcaceae	*Succiniclasticum*
OTU-105	0.37%	0.41%	0.09%	0.05 ± 0.09%	Erysipelotrichaceae	*Asteroleplasma*
OTU-28	6.47%	4.73%	0.09%	0.37 ± 0.70%	Erysipelotrichaceae	*Sharpea*
OTU-68	0.12%	0.15%	0.17%	0.11 ± 0.07%	Erysipelotrichaceae	
OTU-2056	0.10%	0.06%	0.95%	0.57 ± 0.27%	Lachnospiraceae	*Butyrivibrio*
OTU-45	1.18%	0.66%	0.36%	0.05 ± 0.10%	Lachnospiraceae	*Roseburia*
OTU-30	1.95%	1.36%	0.24%	0.03 v 0.06%	Lachnospiraceae	
OTU-20	1.95%	1.41%	0.41%	0.15 ± 0.26%	Ruminococcaceae	*Ruminococcus*
OTU-1880	0.11%	0.20%	0.65%	0.13 ± 0.16%	Ruminococcaceae	*Ruminococcus*
OTU-55	0.12%	0.10%	0.11%	0.16 ± 0.24%	Ruminococcaceae	
OTU-44	0.07%	0.19%	0.20%	0.21 ± 0.17%	Succinivibrionaceae	*Succinivibrio*
OTU-71	0.23%	0.54%	0.01%	0.04 ± 0.12%	Succinivibrionaceae	*Succinivibrio*
Unique OTUs	Detected only in samples from donor and acceptor cow 3 on day 1 and 2 after transfer					
OTU-362	0.09%	0.10%	0.02%	0.02 ± 0.03%	Bifidobacteriaceae	*Bifidobacterium*
OTU-613	0.12%	0.17%	0.01%	0.01 ± 0.02%	Bifidobacteriaceae	*Bifidobacterium*
OTU-2301	0.64%	0.60%	0.00%	0.00 ± 0.00%	Prevotellaceae	*Prevotella*
OTU-394	0.40%	0.18%	0.00%	0.00 ± 0.02%	Prevotellaceae	*Prevotella*
OTU-267	0.42%	0.05%	0.00%	0.00 ± 0.00%	Prevotellaceae	*Prevotella*
OTU-2372	0.30%	0.13%	0.00%	0.00 ± 0.00%	Acidaminococcaceae	*Acidaminococcus*
OTU-553	0.25%	0.08%	0.00%	0.00 ± 0.00%	Acidaminococcaceae	*Acidaminococcus*
OTU-174	0.34%	0.32%	0.00%	0.08 ± 0.03 %	Erysipelotrichaceae	
OTU-455	0.11%	0.03%	0.00%	0.00 ± 0.00%	Family XIII	*Incertae Sedis*
OTU-206	0.37%	0.20%	0.01%	0.01 ± 0.01%	Lachnospiraceae	*Incertae Sedis*
OTU-113	0.09%	0.11%	0.18%	0.08 ± 0.07%	Lachnospiraceae	*Oribacterium*
OTU-40	1.50%	0.55%	0.06%	0.02 ± 0.02%	Lachnospiraceae	*Oribacterium*
OTU-1743	0.22%	0.15%	0.03%	0.01 ± 0.01%	Lachnospiraceae	*Pseudobutyrivibrio*
OTU-498	0.11%	0.06%	0.01%	0.00 ± 0.00%	Lachnospiraceae	*Shuttleworthia*
OTU-1846	0.11%	0.06%	0.05%	0.03 ± 0.04%	Lachnospiraceae	
OTU-480	0.39%	0.33%	0.00%	0.00 ± 0.00%	Lactobacillaceae	*Lactobacillus*
OTU-325	0.57%	0.73%	0.00%	0.00 ± 0.00%	Streptococcaceae	*Streptococcus*
OTU-432	0.36%	0.21%	0.00%	0.00 ± 0.00%	Veillonellaceae	*Megasphaera*
OTU-1098	0.65%	0.70%	0.00%	0.00 ± 0.01%	Veillonellaceae	*Megasphaera*
OTU-5	9.99%	11.21%	0.00%	0.05 ± 0.23%	Veillonellaceae	*Megasphaera*
OTU-998	0.13%	0.13%	0.00%	0.00 ± 0.00%	Veillonellaceae	*Mitsuokella*
OTU-136	0.13%	0.05%	0.03%	0.06 ± 0.04%	Veillonellaceae	*Schwartzia*
OTU-2257	0.36%	0.16%	0.04%	0.01 ± 0.01%	Veillonellaceae	*Selenomonas*
OTU-92	1.39%	0.59%	0.08%	0.02 ± 0.03%	Veillonellaceae	*Selenomonas*
OTU-259	0.73%	0.48%	0.01%	0.01 ± 0.01%	Veillonellaceae	
OTU-228	0.07%	0.17%	0.00%	0.01 ± 0.01%	Campylobacteraceae	*Campylobacter*
OTU-366	0.04%	0.25%	0.01%	0.01 ± 0.01%	Anaeroplasmataceae	*Anaeroplasma*
SUM	**94.5%**	**95.4%**	**16.6%**	**13.2%**		

*average over all samples, but excluding the samples collected on day 1 and 2 from donor and acceptor cow 3

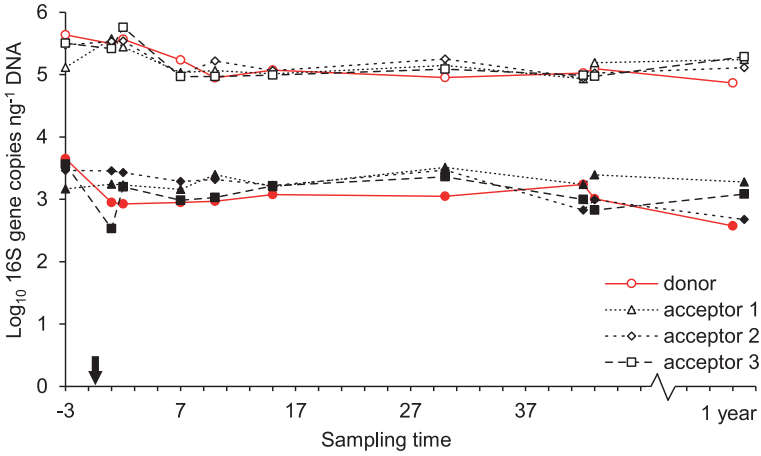


Figure 4.6 Absolute quantity of bacteria (**open symbols**) and methanogens (**closed symbols**) at different time points, expressed as 16S gene copies per ng DNA extract. The arrow indicate the moment of the rumen content transfer (i.e. day 0).

4.4 DISCUSSION

Weimer et al. (2010) was the first to investigate the stability and host specificity of the rumen bacterial community following a near complete exchange of rumen contents between two pairs of cannulated cows. Using ARISA fingerprinting of the bacterial community, the authors determined that the community re-established a profile that resembled the original profile from before the exchange [151]. Similarly, our experiment was designed to investigate the extent to which the host influences the establishment of the rumen microbial community. Compared to Weimer et al. (2010), the current study aimed to remove the rumen content of the donor as completely as possible and the rumen wall was rinsed to remove residual fluid and fibers. Furthermore, metabarcoding was used instead of a community fingerprinting technique like ARISA, in order to investigate the taxonomic composition of the rumen communities. The rumen contents from one donor were subdivided into four equal parts and transferred to the donor cow and three acceptor cows. By this setup, identical inoculation conditions were created during transfer, i.e. the microbial community of the donor was present in each of the four experimental cows.

Despite the fact that each cow received a quarter of identical rumen content of the donor cow in exchange for their whole rumen contents, in the first two days after transfer two distinct responses were observed: (i.) The rumen functioning and feed intake of acceptor cows 1 and 2 were not negatively affected by the rumen transfer and the new rumen bacterial community

composition was mainly influenced by the introduction of a non-indigenous bacterial community and host effects. (ii.) The bacterial community after transfer was influenced by both the introduction of a non-indigenous bacterial community (in the case of the three acceptor cows) as well as a short-term physiological stress and reduced feed intake, which was the case for the donor cow and acceptor cow 3. The physiological stress was presumably due to a severe perturbation of the rumen functioning during the transfer. While removing the rumen contents, the rumen filled with air. The elevated oxygen levels could have disturbed the fermentation activity until anaerobic conditions were restored in the rumen. Furthermore, the emptying and reinoculation with only a quarter of rumen contents might have induced a temperature shock (strengthened by the winter temperatures). The combined effect of these factors might have resulted in physiological stress and reduced feed intake, which translated into major effects on the milk yield, methane production and the microbial community. Fortunately, the samples collected during this period provide a unique inside in the resilience of the rumen microbiome and the way in which the microbial community is restored after a perturbation. In the two days following transfer, the bacterial richness dropped from 1500 to a mere 800 OTUs in the donor cow and acceptor cow 3. While most OTUs remained below detection levels, the remaining bacterial community consisted of a core group of OTUs that was observed in all samples, as well as novel OTUs that gained dominance during the feed intake depression and physiological stress. The overall lower feed intake and the preference towards concentrate resulted in elevated proportions of starch and an increased rumen turnover rate. These conditions imposed selection for bacteria with fast heterofermentative growth, mainly amylolytic species. *Sharpea azabuensis* and *Streptococcus bovis* proliferated under these circumstances, presumably producing lactic acid as primary metabolic end-product [30,337], which in turn induced the growth of lactate-utilizing *Megasphaera elsdenii* [43]. Concomitantly, non-lactic acid producing starch utilizers, *Prevotella bryantii* and *Ruminococcus bromii*, proliferated and may have competed for starch. The competition between non-lactic acid bacteria and lactic acid bacteria, and the interaction between lactic acid-production and consumption is essential in the recovery of the rumen microbial ecosystem during stress. Consequentially, the rumen pH did not decrease below daily averages of 6.4 during the feed deprivation period. The altered microbial community profile, mainly dominated by *M. elsdenii* and *P. bryantii* is reflected in the decreased acetate-to-propionate ratio, the increased proportions of valerate and the seemingly increased feed efficiency. In contrast to the bacterial diversity, qPCR analysis did not indicate a decrease in absolute numbers of bacteria, suggesting that the absolute quantities

of bacteria in the rumen fluid were rapidly restored after transfer (within 24 h) by the rapidly growing bacteria that dominated the rumen community during the feed deprivation period, consuming easily fermentable carbohydrates and stabilizing the ambient conditions of the rumen microbial ecosystem, which primed the way for slow-growing bacteria (cellulolytic species) and cross-feeding species (autogenic successions; Figure S4.4). This response suggests that the rumen ecosystem relies on its vast biosphere of transient and low abundant species to maintain and restore the microbial ecosystem after a severe perturbation.

In contrast to the donor cow and acceptor cow 3, the rumen function of acceptor cows 1 and 2 were only slightly affected by the rumen content transfer, with no observable effect on the methane emissions, milk production, the bacterial and methanogen richness and diversity and only minor short-term changes in the VFA profiles. As a consequence of the experimental design, the rumen microbial ecosystem had to repopulate from a quarter transferred rumen content as inoculum for newly ingested feed, which roughly corresponds to two doublings (from 25 to 50 and 50 to 100, in simplistic terms). The main cultivated rumen bacterial species have doubling times ranging from 1 to 3 h under optimal nutritional and ambient conditions [56,338,339]. Theoretically, the microbial ecosystem should thus be repopulated after approximately 6 h. Indeed, the rumen bacterial profiles of acceptor cows 1 and 2 did not indicate a decrease of richness or diversity during the first days after transfer. The rumen communities of both cows initially maintained the bacterial community composition of the donor cow before transfer. However, this community did not gain a strong foothold and from day 7 and onwards, the community evolved over time, with the donor community as starting point and presumably shaped by host related and external factors.

The variance on the Bray-Curtis dissimilarities of the samples from different time points and different hosts (not taking into account the samples collected during the feed intake deprivation) was small because the main influencing factor, i.e. the diet composition, was standardized before start of the experiment and during the experimental period. After a one week stabilization period following the rumen content transfer, where the donor cow and acceptor cow 3 recovered from a feed intake depression and acceptor cows 1 and 2 initially adopted the rumen microbial profile of the donor, the four cows re-established a new dynamic steady-state community profile, that neither resembled the bacterial profile of the donor nor the original profile of the host prior to the rumen content transfer. Instead, the OTU abundance profiling suggests that the community consisted of a stable core community that was consistently present throughout the

experiment. The core OTUs were complemented by a large group of transient OTUs and throughout the experimental period, different groups of OTUs transiently became dominant in successive phases (allogenic successions; Figure S4.4). Presumably these allogenic successions were driven by external factors (changes in lactation stage, variation in ambient conditions, rumen pH and oxygen levels, stress) and predation [340,341]. Furthermore, the conditions of the rumen ecosystem are strongly influenced by rumen motility, rumination, outflow to the omasum and exchange of water and solutes (through saliva excretion, epithelial absorption and urea-N conservation). In this manner, the host might also exert an influence on the bacterial activity and possibly the community composition.

The bacterial community is resilient because of its high richness and functional redundancy. Although the community itself is dynamic at a taxonomic level, it maintains its functional stability. In contrast to the complexity of the bacterial community, the methanogen community is represented by only a few dominant OTUs that were detected in the samples from each time point and each cow. The methanogen community is thus characterised by a very low richness and diversity, but a stable composition over time. The methanogen community was unaffected by host effects or time effects. Within the rumen ecosystem, the methanogens are specialised in consuming H_2. As such they occupy a very specific and ever-present niche for which they do not have any significant competition. Opposite to the rumen bacterial communities, with very dynamic and influenceable compositions due to their functional redundancy, the rumen methanogens enjoy a high degree of phylogenetic stability. But though phylogenetic shifts are mostly absent, the functionality of the methanogen communities is more sensitive and variable. This is evidenced by the feed intake depression of the donor cow and acceptor cow 3, which resulted in a diminished availability of H_2 and consequently lower methane emissions. But even under these conditions, the methanogens maintained a stable population in terms of absolute abundances and taxonomic profile.

4.5 CONCLUSION

Following the introduction of a non-indigenous community, the bacterial community composition reached a new dynamic equilibrium and consisted of a stable core community and temporary dominant species who were continuously succeeded by species from the large pool of transient and subdominant species. Opposite to the bacteria, the methanogen community proved more resilient against stress despite their low diversity. The methanogen community

composition and absolute abundance proved resilient even during severe perturbations of the rumen microbial ecosystem, however, the methanogenesis of the community was reduced during decreased feed intake.

4.6 SUPPLEMENTARY DATA

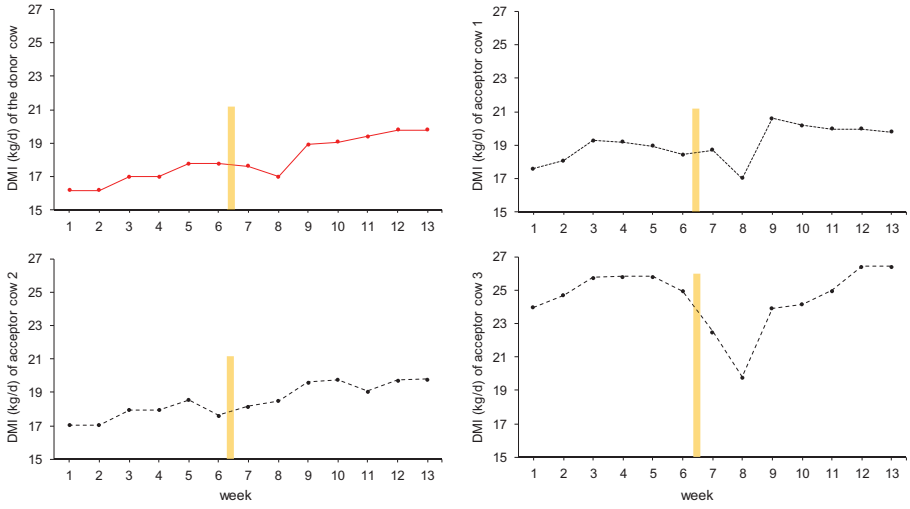


Figure S4.1 weekly averages of daily dry matter intake (kg DMI per day). The yellow bars represent the moment of rumen content transfer.

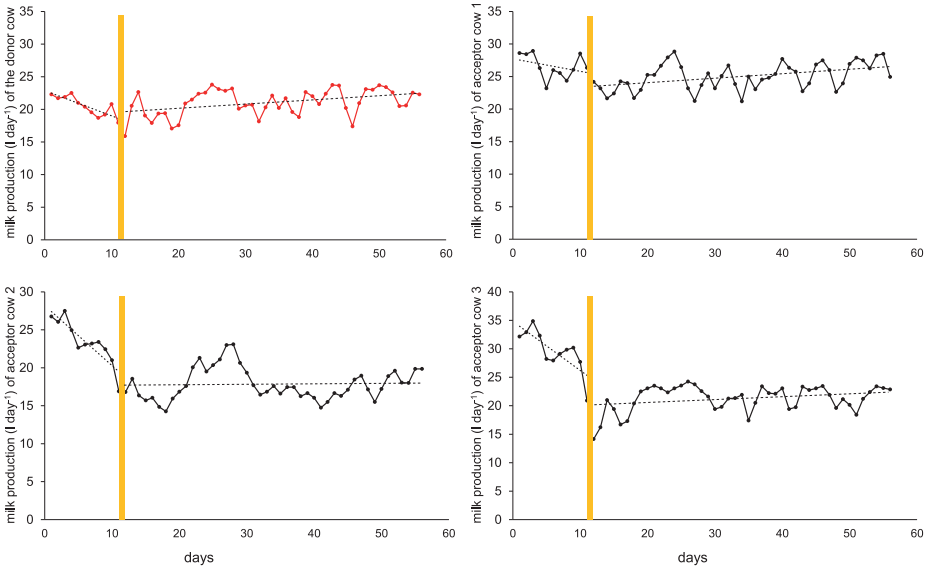


Figure S4.2 Moving averages (per two days) and linear trend line of daily milk production (liter per day) of each cow. The Yellow bars represent the moment of rumen content transfer.

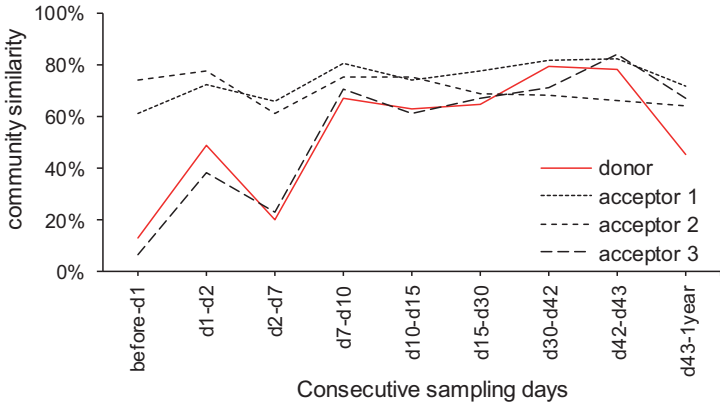


Figure S4.3 Moving window analysis of the community similarity (1 – Bray-Curtis dissimilarity) between consecutive sampling days.

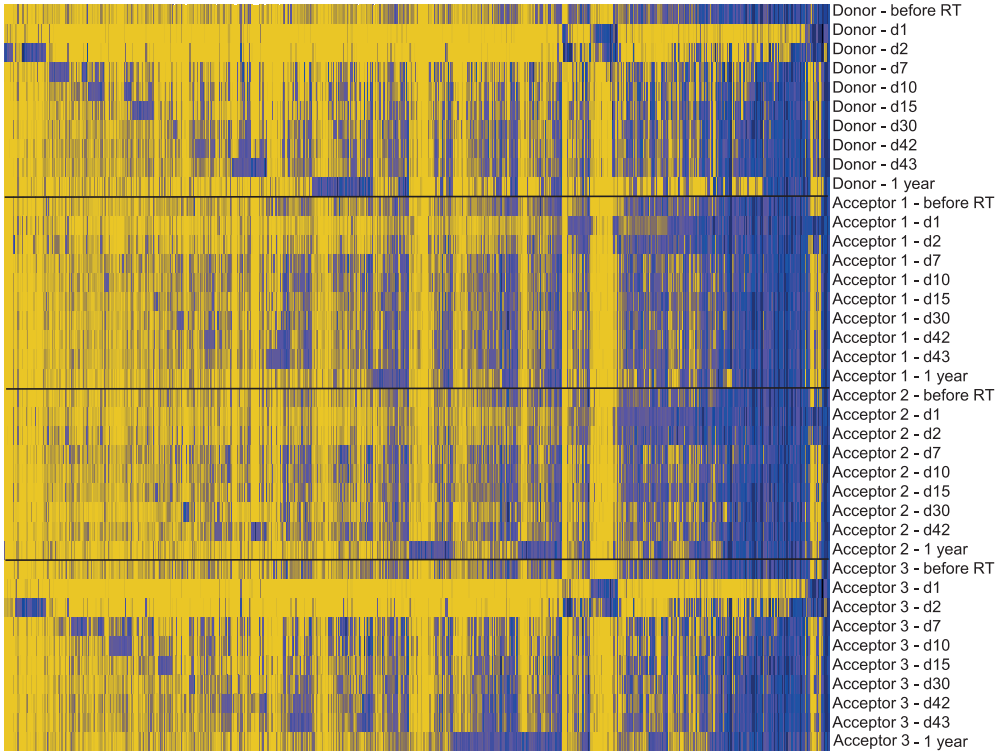


Figure S4.4 Heatmap of the bacterial OTUs (horizontal) with prior sample wide abundance sorting. The samples (vertical) are positioned per individual cow (delineated by a black line) and chronological.

Chapter 5

The effect of residues of doxycycline, due to cross-contamination of feed, on the microbial community in pig's feces

CHAPTER 5 THE EFFECT OF RESIDUES OF DOXYCYCLINE, DUE TO CROSS-CONTAMINATION OF FEED, ON THE MICROBIAL COMMUNITY IN PIG'S FECES

Abstract

Residues of doxycycline can unintentionally carry-over from a medicated feed to non-target feeds, resulting in the subtherapeutic administration of medication to pigs. This study investigates the influence of a carry-over of 3% of the therapeutic dose of doxycycline hyclate (DOX), on the bacterial community in the feces of pigs, focusing on the taxonomic composition and the abundance of specific tetracycline resistance genes.

Doxycycline reached a stable concentration of about 4 mg kg^{-1} in the feces of treated pigs after four days of feeding the "contaminated" diet. Concomitantly tetracycline resistance genes *tet*(W) and *tet*(L) significantly increased, whereas other tested resistance genes *tet*(O), *tet*(Q), *tet*(A), *tet*(M), *tet*(B) were not enriched during treatment. The fecal microbial community composition seemed unaffected by the continuous influx of subtherapeutic doxycycline and no taxonomic groups were significantly enriched during DOX treatment, as compared to the control group, who did not receive DOX. Only a short-term effect was observed on the microbial richness and diversity, which was lowest on the fourth day of administration.

The carry-over of 3% of a therapeutic dose of DOX did not seem to induce the enrichment of most of the tested resistance genes nor influence the composition of the fecal microbial communities of pigs.

5.1 INTRODUCTION

Doxycycline is a semi-synthetic tetracycline derivative. As hyclate salt, doxycycline hyclate is frequently used to treat or prevent respiratory infections caused by common porcine pathogens (*Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*) [342,343]. According to the Belgian Veterinary Surveillance of Antibacterial Consumption, doxycycline represented 17.6% of the antibacterial use in Belgian veterinary medicine in 2016 [156], despite its high importance for human medicine [344]. Doxycycline hyclate (DOX) is often administered to pigs as additive in medicated feedstuffs. Medicated feed are produced in the feed mill by mixing an antibiotic compound in the feed, before being transported to the farm, stored in silos and administered to the target herd. During the different steps of this process, antibiotic residues from the medicated feed can transfer to non-target feeds [159]. These events of cross-contamination can be limited by using end-of-line mixing at the feed mill and fine dosing systems on trucks. Despite these efforts, cross-contamination remains a topical problem.

Peeters et al. (2016) designed and executed an *in vivo* experiment to determine the fecal and intestinal concentrations of antibiotics (doxycycline, chlortetracycline and sulfadiazine-trimethoprim) when pigs are exposed to 3% of a therapeutic dose in the feed [173]. Fecal samples were collected and antibiotic residues were quantified using LC-MS analysis. During continuous administration, doxycycline reached a concentration of about 4 mg kg^{-1} in the feces of the treated pigs [173]. In the current study, the collected feces was used to investigate the effect of these subtherapeutic concentrations of DOX on the fecal microbiome and the abundances of specific tetracycline resistance genes.

5.2 MATERIAL AND METHODS

5.2.1 *In vivo* experiment

The *in vivo* experiment was carried out at the Veterinary and Agrochemical Research Centre (CODA-CERVA). The experimental design, the feed composition and sampling techniques were thoroughly described by Peeters et al. (2016) [173]. In short, two groups of each six pigs were housed in a separated pen. The control group (CTRL) received a regular experimental diet whereas the treatment group (TREAT) received the same diet but with 3% of a therapeutic dose of DOX (i.e. 3% of 13 mg kg^{-1} DW day^{-1}), corresponding to 9.98 ± 5.35 mg DOX kg^{-1} feed.

Individual fecal samples were collected from pigs using rectal stimulation, on 6 sampling days: just before administrating the medicated feed (day -1) and during treatment on day 2, 4, 6, 8 and 10. Immediately after collection, samples were stored at -80°C.

5.2.2 DNA extraction

Fecal samples were defrosted and homogenized. DNA extractions were carried out as described in Chapter 2, section 2.2.2. For 8 samples, insufficient fecal matter was collected for reliable DNA extraction and were left out for further analysis.

5.2.3 Library preparation and metabarcoding

Amplicon sequencing of the bacterial V3-V4 variable region of the 16S rRNA gene was performed on 64 samples. The library preparation was performed using the Illumina protocol for 16S metagenomic sequencing [252], similarly as described in Chapter 2, section 2.2.3. The final barcoded library was sequenced on a Illumina MiSeq (PE 2x300). The processing of the sequenced reads was described in Chapter 2, section 2.2.4, with minor changes. Instead of using the Usearch "fastq_mergepairs" command, the forward and reverse reads were merged using PEAR 0.9.8 with a minimum overlap length of 120 bp, a minimum and maximum resulting length of 400 bp and 450 bp and a quality threshold of 30 with a minimum length of 200 bp after trimming [256]. Processing the data resulted in an average library size of 99 798 reads per sample. Rarefaction analysis was done to ascertain that the library size of each sample was sufficient to analyze the bacterial community. Simpson diversity and observed richness were calculated with the Phyloseq package in R [264]. For subsequent data analysis, only OTUs representing at least 0.1% of the total community in at least one sample were retained, reducing the total number of OTUs from 1528 to 524 (still representing 97% of the reads, on average). This OTU table was used to pairwise Bray-Curtis dissimilarity indices and community differences between samples were visualized with nonmetric multidimensional scaling (NMDS) using the Vegan package in R [263].

5.2.4 Quantitative PCR

QPCR analysis was performed on a LightCycler® 480 Real-time PCR system (Roche). Total 16S rRNA gene abundance, as a proxy for bacterial abundance, was quantified using SYBR Green technology. For each DNA extract, a 1000-fold dilution was made and analyzed in duplicate, with a reaction mixture and PCR conditions described in Desloover et al. (2015)

[345]. The abundances of five tetracycline resistance genes *tet*(Q), *tet*(O), *tet*(M), *tet*(W) and *tet*(B) were quantified using TaqMan qPCR assays, using a 10 or 100-fold dilution of the DNA extracts. Each reaction mixture contained, in a total volume of 25 µl, 12.5 µl TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA), 300 nmol l^{-1} of each primer, 100 nmol l^{-1} probe and 5 µl of template DNA. The PCR program was carried out in a thermal cycling process consisting of a hot start activation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The primers (and probes) and corresponding annealing temperatures and PCR efficiencies are summarized in Table 5.1. Within each run, a standard curve was constructed using a 10-fold dilution series of plasmid DNA (IDT, Coralville, IA, USA) to determine PCR efficiency. The total number of gene copies was calculated by converting the quantification cycle values (Cq) to gene copy abundances, using the standard curve and taking the PCR efficiency into account.

Table 5.1 Primers and probes used for qPCR in this study

Primers	target	sequence (5' – 3')	T_a	ref.	efficiency
Bac338_F Bac518_R	16S rRNA Bacteria	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	60°C	[269]	94.21%§
tetQ-F tetQ-Taq* tetQ-R	*tet*(Q) gene	AGGTGCTGAACCTTGTTTGATTC TCGCATCAGCATCCCGCTC GGCCGGACGGAGGATTT	60°C	[346]	96.26%§
tetO-F tetO-Taq* tetO-R	*tet*(O) gene	AAGAAAACAGGAGATTCCAAAACG ACGTTATTTCCCGTTTATCACGG CGAGTCCCCAGATTGTTTTTAGC	60°C	[346]	95.53%§
tetM-F tetM-Taq* tetM-R	*tet*(M) gene	GGTTTCTCTTGGATACTTAAATCAATCR ATGCAGTTATGGARGGGATACGCTATGGY CCAACCATAYAATCCTTGTTCRC	60°C	[346]	91.60%§
tetW-F tetW-Taq* tetW-R	*tet*(W) gene	GCAGAGCGTGGTTCAGTCT TTCGGGATAAGCTCTCCGCCGA GACACCGTCTGCTTGATGATAAT	60°C	[346]	98.52%§
tetB-F tetB-Taq* tetB-R	*tet*(B) gene	ACACTCAGTATTCCAAGCCTTTG AAAGCGATCCCACCACCAGCCAAT GATAGACATCACTCCCTGTAATGC	60°C	[346]	99.25%§
tetL_F tetL_Taq* tetL_R	*tet*(L) gene	GGTTTTGAACGTCTCATTACCTGAT CCACCTGCGAGTACAAACTGGGTGAAC CCAATGGAAAAGGTTAACATAAAGG	60°C	[346]	90.77%§
tetA_F tetA_Taq* tetA_R	*tet*(A) gene	CCGCGCTTTGGGTCATT TCGGCGAGGATCG TGGTCGCGTCCCAGTGA	60°C	[347]	92.58%§

* The TaqMan probes were dual-labelled with 5'-FAM (fluorescein) and 3'-BHQ1 (Black Hole Quencher®)

§ PCR efficiency of each assay were quantified with a dilution series of the commercial vector pIDTSMART_AMP (IDT, Coralville, Iowa, USA), including the target sequence of the 16S gene fragment of *Prevotella ruminicola* (NCBI: NC_014033) or including the concatenated target sequences of each tested resistance gene, flanked by "TATA". The target sequences were obtained from NCBI: JQ966986.1 for *tet*(B), KF408178.1 for *tet*(M), M18896.2 for *tet*(O), X58717.1 for *tet*(Q), AF202986.1for *tet*(W) and JQ280488.2 for *tet*(L).

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of subtherapeutic DOX on the *in vivo* community

Statistical analysis did not reveal a significant effect of DOX administration on the richness or Shannon-Wiener diversity, using a generalized linear mixed effects model with group (treatment versus control), sampling time and their interactions as fixed effects and host animal as random effect. Subsequent *post-hoc* testing using Tukey adjustment, indicated that the alpha-diversity values of the control animals did not significantly differ from the treated animals per sampling day. If only the treated group is considered and time points during administration (day 2, 4, 6, 8 and 10) are compared to the pretreatment period (day -1) using two-sample t-test, only day 4 appears significantly different from the pretreatment period ($p = 0.026$). This means that the administration of 3% of a therapeutic dose of DOX in the feed of pigs only had a short-term influence on the fecal bacterial richness and diversity. After the fourth day of treatment, when DOX residues reached a steady-state concentration of 4 mg kg^{-1} in the fecal samples, the richness was at its lowest (the Shannon diversity numerically). In subsequent days, alpha-diversity values began to recover and reinstated the pretreatment levels (Figure 5.1).

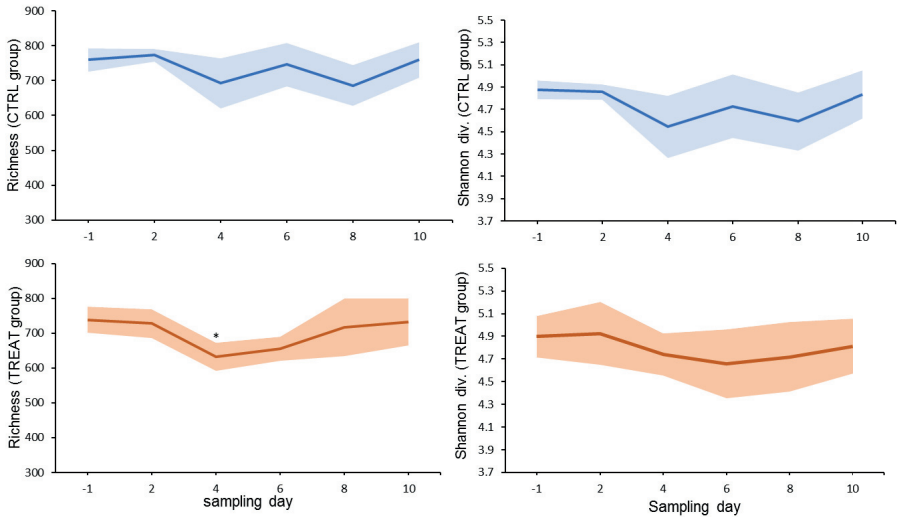


Figure 5.2 Plot of the mean richness, i.e. the numbers of OTUs (**left**), and mean Shannon-Wiener diversity indices (**right**) with the 95% confidence intervals, measured by metabarcoding in samples collected from the control pigs (**blue**) and the pigs receiving 3% of a therapeutic dose of DOX (**orange**).

Despite small influences on the bacterial richness, no effects were observed on the community composition. The random positioning of samples in the NMDS plot suggest that the subtherapeutic concentrations of DOX did not have a significant effect on the fecal bacterial communities (at OTU level), instead the microbial composition seemed to be influenced by host animal and sampling moment (Figure 5.2). The fecal microbial communities are similar between tested animals due to equal diet composition, physiological state and living conditions, but the observed differences amongst taxonomic profiles could be attributed to host specific factors influencing the microbiome, as well as the dynamic nature of the microbiome over time. The dynamics of the fecal bacterial communities was monitored by measuring variations in the community similarities between sequential sampling times, using the Bray-Curtis dissimilarity indices. The moving window analysis suggests that fecal bacterial communities changed on average 28 ± 7% between consecutive samples from the same host (Figure 5.3). In this complex and fluctuating background, it is difficult to distinguish possible taxonomic changes due to DOX administration. This is further complicated as the intestinal microbial communities of different hosts may not respond uniformly to the presence of a broad-range antibiotic with bacteriostatic effects, such as doxycycline.

The abundances of 16S rRNA gene copies as a proxy for bacterial densities and the abundances of specific tetracycline genes were quantified with qPCR assays. Pigs were randomly assigned to the TREAT or CTRL group. Nevertheless, the fecal microbial communities of the TREAT group had a higher bacterial load as compared to the CTRL group. To be able to compare different samples, the abundances of the *tet* genes are reported relative to the total 16S rRNA gene copies. Preliminary analysis of the pig's intestinal resistome during DOX treatment by whole genome shotgun sequencing identified *tet*(Q), *tet*(O) and *tet*(W) as most dominant determinants of tetracycline resistance (data not shown). These genes are often reported as major representatives of tetracycline resistance in agricultural ecosystem [348–351] and were therefore quantified in the fecal samples, complemented by the detection of other common tetracycline resistance determinants in the intestinal ecosystem: *tet*(A), *tet*(B), *tet*(M) and *tet*(L) [348,351,352].

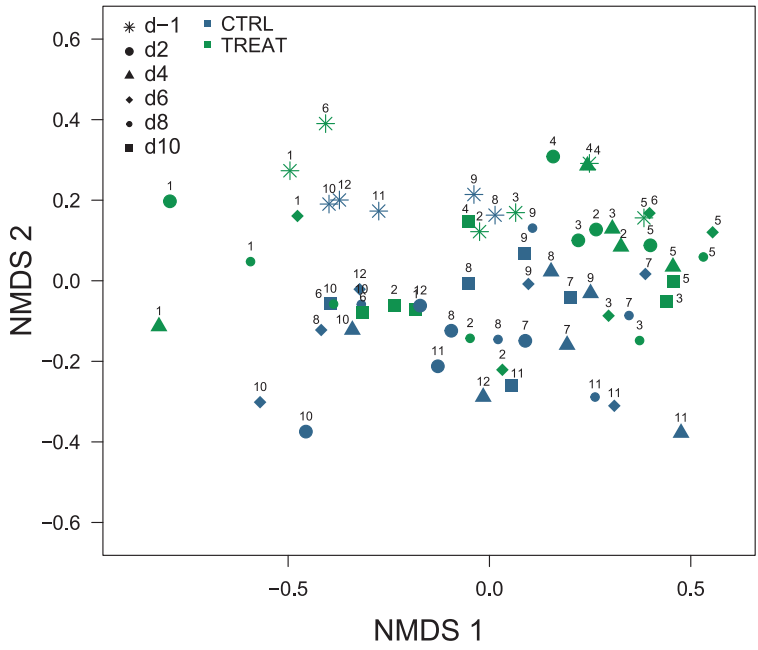


Figure 5.2 NMDS profile of pairwise community dissimilarity (Bray−Curtis) indices of bacterial 16S sequencing data (0.1% cut off) of fecal samples collected from 6 control pigs (CTRL, animals 7 to 12) and pigs exposed to 3% of a therapeutic dose of DOX (TREAT, animals 1 to 6) on 6 sampling days (before treatment (-1), day 2, 4, 6, 8, 10). The host identification number is indicated with each sample point

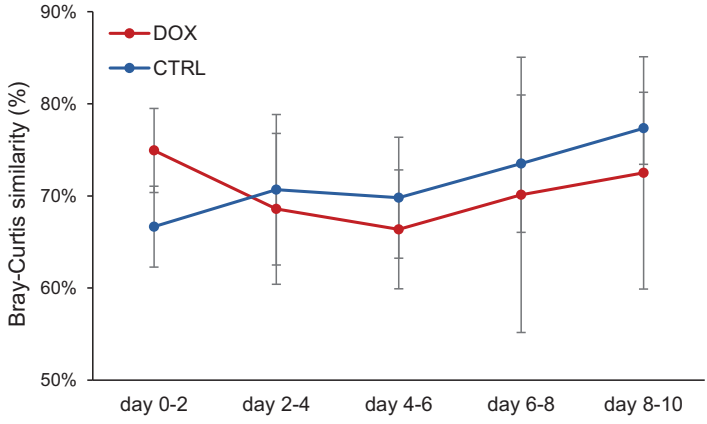


Figure 5.3 Moving window analysis of the community similarity based on Bray-Curtis indices (1-BC %) between consecutive sampling days

Even in the absence of any antibiotic treatment, the feces of the pigs in the CTRL group contained high numbers of *tet* genes: *tet*(Q) genes were present at an average of 0.4%, *tet*(O) and *tet*(W) both represented 1.7% (i.e. number of *tet* genes per hundred 16S rRNA genes). Within the TREAT group, *tet*(W) abundances significantly increased ($p = 0.01$) by a factor 1.5 from day 4 an onwards, as compared to the samples from day -1 and day (using two-sample t-test). This represented an increase from 2.2% to 3.0% or an addition of 4.3 $x10^5$ *tet*(W) genes/ng DNA. Noticeably, abundances of *tet*(W) increased simultaneous with the doxycycline concentrations in the feces, which only reached steady-state concentrations on day 4. Also *tet*(L) gene copies significantly increased ($p = 0.0003$) during subtherapeutic DOX treatment. Contrary to *tet*(W), *tet*(L) increased linearly from 0.02% on day -1 to 1.6% on day 10, representing an addition of 6.5 $x10^5$ *tet*(L) genes/ng DNA. The abundances of other *tet* genes did not significantly increase or decrease during DOX administration (Figure 5.4). Presumably, the fluctuations in abundances of tetracycline resistance genes are due to fluctuating abundances of several genera amongst the different hosts and between different sampling days, which could be the reason for the high deviation observed on the mean numbers of specific *tet* genes. Similarly, the host specificity of the fecal microbial community could be the reason for the observed differences in concentrations of specific *tet* genes. The gastrointestinal ecosystem of the animals in the CTRL group might contain more taxa with *tet*(O), whereas the animals in the TREAT group might have higher abundance of taxa encoding *tet*(Q) and/or *tet*(W). Though the qPCR data could not be positively correlated with the community profiles.

The increase of specific tetracycline-resistance genes is correlated with the absolute abundances of specific species or taxa in the microbial community. The resistance determinant *tet*(L) catalyzes the efflux of divalent tetracycline-metal complexes in exchange for protons. The presence of *tet*(L) has been demonstrated, amongst others, in isolates from the *Bifidobacteria* [353], *Lactobacillus* [354,355] and *Streptococcus* [356,357], dominant genera of the pig's fecal microbiome (representing 0.43 ± 0.51%, 9.2 ± 3.3% and 6.3 ± 4.7%, respectively). The resistance gene *tet*(W) codes for ribosomal protection proteins, and has broad taxonomic distribution in both Gram-positive and Gram-negative bacteria, including *Megasphaera* [358], *Butyrivibrio* [359,360], *Lactobacillus* [354,355] and even certain isolates from the genera of *Prevotella*, *Veillonella* and *Streptococcus* [361]. *Tet*(W) likely owes its dominance and omnipresence in the pig's intestinal microbial ecosystem to its broad taxonomic distribution in the genera dominating these ecosystems. The increased gene copies of *tet*(L) during DOX

administration may be a consequence of the concomitant proliferation of streptococcus, a genera with isolates containing *tet*(L). Streptococci, represented by two OTUs in the metabarcoding data, increased from 0.36 ± 0.39% (day -1) to 5.6 ± 2.7 (days 4-10) in the animals receiving DOX. Furthermore, the relation of *tet*(L) and streptococci was further suggested during the *in vitro* trials (described in Chapter 6), where a rapid decline in relative abundances of *Streptococcus* (from about 0.15% to below detection levels at day 2) coincided with a similar decline in *tet*(L) concentrations (from about 300 copies ml^{-1} to near 0). The increase of *tet*(W) during antibiotic administration is more difficultly attributed to any specific genera. Other than *Streptococcus*, no genera uniformly increased or decreased during DOX administration in the fecal microbiome of the tested pigs. Strikingly, *tet*(M) and *tet*(A) gene copies decreased during the *in vivo* experiment, but this reduction was observed in both the CTRL and the TREAT group.

The qPCR and metabarcoding results highlight the shortcomings of these assays and emphasize their limitations. The number of tetracycline resistance genes is related to the absolute numbers of certain species, but metabarcoding is semi-quantitative at best and the observed increases/decreases in relative abundances might not represent increases/decreases in absolute numbers of taxa. Alternative assays could perhaps better estimate the potential of doxycycline administration on resistance development. For example, classic plate cultivation in the presence and absence of doxycycline (in the agar) can be an addition to the above mentioned techniques to identify and enumerate resistance and total cultivable species. Furthermore, the experimental design is prone to drawbacks inherently linked to *in vivo* experimentation. The intestinal and fecal microbiome were not exposed to stable concentrations, but instead the levels of doxycycline may have varied due to the suboptimal homogeneity of DOX in the feed and the diurnal patterns of feeding. The intestinal microbial composition is shaped by host-related factors and the effect of DOX on the taxonomic composition may thus be host specific, making it difficult to compare biological repeats. Although the experimental design provides an accurate idea of the influence of cross-contamination of doxycycline under actual farm conditions. Nevertheless, it would be interesting to investigate the effect of such subtherapeutic concentrations under controlled conditions: repeatable intestinal microbial ecosystems, continuously exposed to a stable concentration of doxycycline.

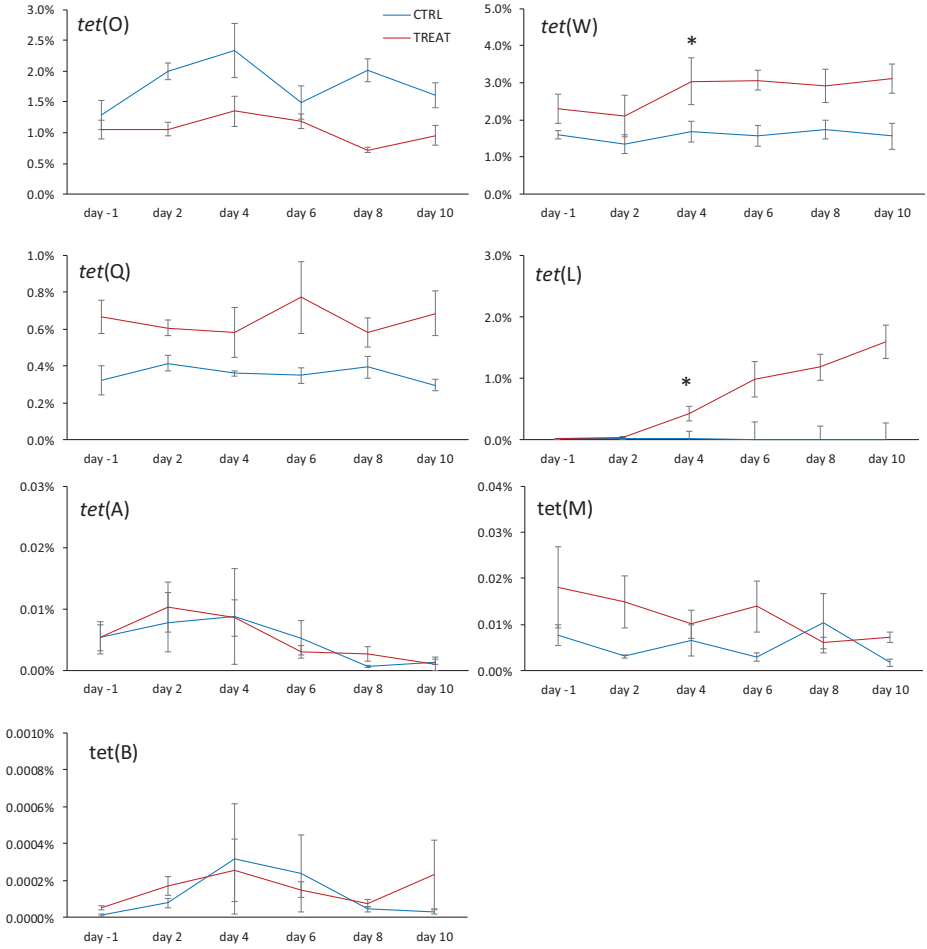


Figure 5.4 Mean resistance gene copy number (and standard error) *tet*(W, O, Q, L, M, B, A) in samples from pigs in the control group (CTRL) and the pigs treated with 3% of a therapeutic dose of DOX (TREAT), normalized to ambient 16S rRNA gene levels and expressed in percentages.

Chapter 6

Impact of subtherapeutic concentrations of doxycycline on the microbial ecosystem in an *in vitro* model of the pig's cecum

CHAPTER 6 IMPACT OF SUBTHERAPEUTIC CONCENTRATIONS OF DOXYCYCLINE ON THE MICROBIAL ECOSYSTEM IN AN IN VITRO MODEL OF THE PIG'S CECUM

Abstract

Cross-contamination of feed with antibiotics causes pigs to become unintentionally exposed to subtherapeutic concentrations of antibiotics. This study investigates the effect of such antibiotic residues of doxycycline hyclate (DOX) in an *ex vivo* model of the intestinal tract of pigs, focusing on the microbial community, microbial activity and the enrichment of resistant bacteria and resistance genes.

The effect of three concentrations DOX were tested; 1 and 4 mg l^{-1} (which correspond to the intestinal concentrations when pigs are fed a compound feed containing 3% of a therapeutic dose) and a reference concentration of 16 mg l^{-1}. The tested concentrations of doxycycline were continuously administered to a chemostat, simulating the microbial ecosystem of the pig cecum and inoculated with cecal content of organically grown pigs. The reactors were initially operated with regular feed medium to obtain a control period against which the effect of the continuous doxycycline administration was compared. The administration of even the lowest DOX concentration caused a significant decrease in bacterial activity, while the microbial community profile seemed to remain unaffected by any of the concentrations. A concentration of 1 mg l^{-1} DOX caused minor selection pressure for tetracycline resistant *E. coli* but not for other groups enumerated with plate cultivation, while 4 mg l^{-1} induced major enrichment of tetracycline resistant *E. coli*, Enterobacteriaceae and total anaerobes. High abundances of *tet*(Q), *tet*(M), *tet*(W), *tet*(O) and *tet*(B) were detected in the inoculum and also before antibiotic administration in the chemostat and did not significantly increase during administration of 1 and 4 mg l^{-1}DOX. Only 16 mg l^{-1} DOX caused minor enrichments. As a second research goal, the *in vitro* microbial community and activity was compared to its *in vivo* counterpart (i.e. the pig's cecum) to evaluate the reactor as simulation of the pigs cecum.

The *in vitro* simulation proved an appropriate model for the microbial ecosystem of the pig's cecum. Subtherapeutic concentrations of doxycycline, as a result of cross-contamination, cause a selection pressure for resistant bacteria and negatively affect microbial activity.

6.1 INTRODUCTION

Intensive pig farming has one of the highest uses of antimicrobial therapy in the agricultural sector [362,363]. Increasing bacterial resistance and the emergence of multi-resistant strains has brought awareness to the dangers of frequent antibiotic use. In response, the European Commission created legislative restrictions on antibiotic use by regulating administration [364,365] and banning the use of antimicrobial feed additives as growth promoting agents [153], thereby restricting subtherapeutic antibiotic treatments. Despite these efforts, antibiotic use in the pig industry remains high. Callens et al. (2012) determined that 98% of the surveyed pig herds in Belgium still received prophylactic antibiotic treatment in 2010 [157]. Between 2011 and 2015, The Belgian Veterinary Surveillance of Antimicrobial Consumption reported an average annual production of 53 tons of antimicrobial premixes for Belgian veterinary medicine, of which 99.6% was intended for medicated pig feed [366]. During production, processing, transport and storage of these medicated feed, trace concentrations of the active antimicrobial compound may transfer to non-medicated feed. This carry-over is known as "cross-contamination". Dutch researchers investigated the magnitude of antibiotic cross-contamination in the pig industry. Fecal samples of fattening pigs (n = 340), who did not receive medical treatments 60 days prior to slaughter, were collected at the abattoir. 55% of the pigs tested positive for at least one antibiotic. Nine antibiotics were detected in total, of which doxycycline was the main representative as it was detected in 31% of the samples [161]. The magnitude of the risk of cross-contamination of feed with a specific antibiotic depends on the frequency of use, the manner of production and administration and the half-life of that antibiotic. Different types of antimicrobial formulations can be used for group treatment. The sales of oral solutions (19.6%), oral powders (33.7%) and premixes (38.2%) accounted for the majority of veterinary antibiotics sold in the EU [367], although some countries (Austria, Denmark, Germany, Luxembourg and the Netherlands) show a preference for oral solutions and powders over premixes, whereas the opposite is true for other countries (Bulgaria, Cyprus, Portugal, Spain and the UK). The carry-over of antimicrobial residues from a medicated feed to a non-target feed is an unavoidable problem inherent to the production of medicated feed in feed mills using premixes [368]. Stolker et al. (2013) determined that 87% of flushing feeds (non-medicated feed produced after a medicated feed in a feed mill) tested positive for at least one antibiotic in the range of 0.1-254 mg kg^{-1} [160]. The use of oral solutions and powders, which can be mixed in with the feed or drinking water directly on the farm, can limit cross-

contamination by bypassing the feed mill and transport of medicated feed. However, cross-contamination may still occur at the farm during mixing, storage and administration. Filippitzi et al. (2016) built a risk model to estimate the probability of cross-contamination. Assuming that medicated feed represents 2% of the total annual feed production in a country, the model predicts that 5.5% of the produced feed would be cross-contaminated with various levels of antimicrobial compounds due to practices related to medicated feed. According to their calculations, 29.7% of the cross-contamination is due to carry-over occurring at the feed mill, 35.1% during transport and 35.2% at the farm [159]. These values demonstrate that cross-contamination of feed is a topical and frequent problem, causing pigs to be unintentionally exposed to subtherapeutic concentrations of antibiotics.

Residual amounts of antimicrobial compounds in the feed will pass the intestinal tract before reaching systemic circulation. Concentrations in the gut compartments depend on the initial concentration in the feed, as well as the pharmacokinetic properties and bioavailability of the drug. In an *in vivo* experiment, Peeters et al. (2016) studied the intestinal concentrations of chlortetracycline, doxycycline hyclate and sulfadiazine-trimethoprim when pigs were fed a compound feed including 3% of the maximum recommended dose of the antibiotic. Doxycycline was detected with concentrations in the range of 1 and 4 mg kg^{-1} intestinal content [173]. In this study, we aimed to investigate the effect of these subtherapeutic concentrations of doxycycline hyclate (DOX) on the microbial community, the microbial activity and the resistance development in an *ex vivo* model of the intestinal ecosystem of pigs. Therefore, a chemostat was designed to simulate the microbial ecosystem of the pig cecum. While current *in vivo* studies are restricted by fecal samples or end point sampling after slaughter, the *ex vivo* model provides the opportunity of easy and standardized sampling to study the effect of DOX on the simulated microbial community of the cecum, without interference of host effects and environmental factors that would greatly increase variance in the results. Although many types of antibiotics are used in the farming industry, we specifically focused on DOX as it remains one of the most frequently used antibiotics in pig husbandry [156] and remains persistent and active in different matrices over extended periods. Widyasari-Mehta et al. (2016) determined that DOX has a half-life of 120 days in liquid pig manure and premix manufacturers reported a shelf-life of 3-5 months of medicated feed [369]. The frequent use of DOX is evidenced by the high prevalence of DOX residues detected in pig feces. Chen et al. (2012) reported maximum DOX concentrations of 7.1 mg kg^{-1} in manure collected in four eastern Chinese pig farms [370]. Carballo et al. (2016) detected doxycycline residues with a mean concentration of 1.2 mg kg^{-1}

in 50% of the collected pig manure samples from eight Spanish pig farms [371]. Because of the frequent use of DOX in group treatments, together with its persistence, cross-contamination of feed with DOX seems to be a frequent and topical problem in pig husbandry.

6.2 MATERIAL AND METHODS

6.2.1 *In vitro* simulation of the pig cecum

The physical and chemical characteristics of the pig cecum were simulated with an *ex vivo* continuous fermentation model (Figure 6.1), as previously described by Messens et al. (2010) [372], with minor modifications. The equipment consisted of similar BioFlo 110 and BioFlo 115 bioreactors, with a 1.3 liter fermenter vessel (New Brunswick Scientific, Enfield, CT, USA). Both units were set up in parallel to perform two experimental runs simultaneously. The reactor vessels were filled with 0.5 liter feed medium, autoclaved and brought to a pH of 6.5 and a temperature of 37°C. After inoculation, the reactors were initially operated in batch mode for 24 h, followed by 9 consecutive days of continuous culture (Figure 6.2). Fresh medium was added via a peristaltic pump at a rate of 1.8 ml min^{-1} and reactor content was removed as waste at the same rate to retain a working volume of 0.5 liter and a transit time of 4.6 h, reflecting the nominal residence time of cecal content in pigs. The pH was kept constant at 6.5 with 3 mol l^{-1} NaOH solution and the temperature was maintained at 37°C. The temperature, pH and redox were continuously monitored with sensors (Ingold® pH probe and Ingold® redox probe; Mettler Toledo, Zaventem, Belgium). Anaerobic conditions were maintained by flushing the headspace of the vessels with an anaerobic gas mixture (20% CO_2, 80% N_2) at a flow rate of 20 ml h^{-1}. The reactor content was kept homogeneous through constant agitation (150 rpm). After a 24 h batch incubation period, continuous fermentation with regular feed medium was carried out during 4 days. From day 5 onwards, the regular feed medium was complemented with 1, 4 or 16 mg l^{-1} doxycycline hyclate (DOX) (Fagron, Waregem, Belgium). The concentrations of 1 and 4 mg l^{-1} represent the upper and lower limits of intestinal concentrations in pigs exposed to feed containing 3% of the maximum recommended dose of DOX [173]. In addition, we tested 16 mg l^{-1} DOX as this concentration is above the epidemiological cut-off values (ECOFF) of doxycycline for all species listed in the EUCAST database [374]. This concentration is therefore considered a positive control to investigate a dose that would certainly elicit a response of the wild type (WT) and susceptible species (as well as some of the

resistant species) in the bacterial communities of the bioreactors. This positive control is compared to the two subtherapeutic concentrations.

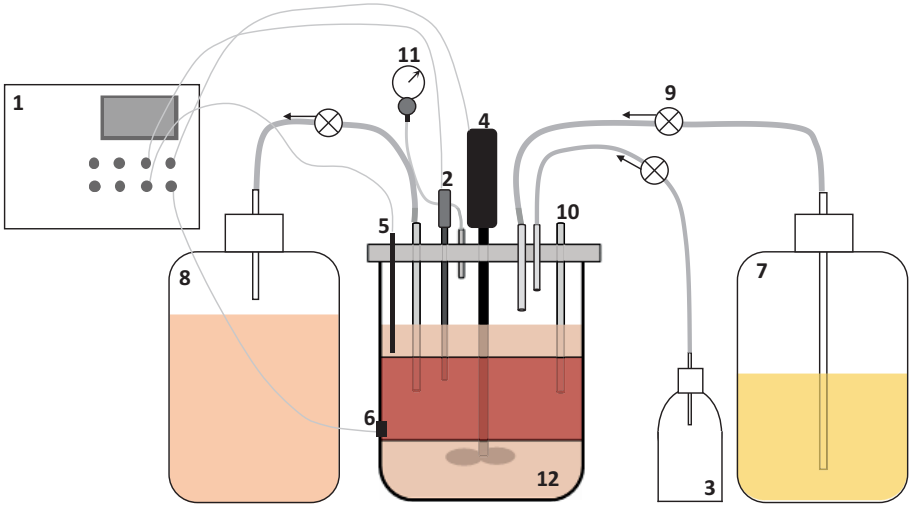


Figure 6.1 Schematic overview of the reactor setup: **1.** Control of pH, temperature and agitation; **2.** pH probe; **3.** 3M NaOH solution. The pH probe registers the real time pH. If the pH drops below 6.5, 3M NaOH is administered to the reactor to ensure a continuous pH of 6.5; **4.** Agitator: flat blade turbine (agitation speed = 120 rpm); **5.** Temperature probe; **6.** Heat blanket. The temperature probe registers the real time temperature. If the temperature decreases, the heat blanket will warm to ensure a continuous temperature of 37°C; **7.** Acidified feed medium kept at 4°C and with continuous magnetic stirring; **8.** Waste collection; **9.** Peristaltic pump. The speed of the waste and feed medium pumps determine the transit time and keep a constant volume of 500 mL inside the reactor; **10.** sample collection tube; **11.** Inflow of a 1:5 mixture of CO_2 and N_2 at 20 ml min^{-1}; **12.** New Brunswick bioreactor vessel (Bioflo 110 or 115).

Three replicate reactor runs were executed for each concentration of DOX. After a four day pretreatment period with regular feed medium, considered as the control period, reactor runs 1, 2 and 3 received 1 mg l^{-1} DOX, reactor runs 4, 5 and 6 received 4 mg l^{-1} DOX and reactor runs 7, 8 and 9 received 16 mg l^{-1} DOX. Ten ml of the reactor content was sampled daily at 9 a.m. for microbial and molecular analysis. Two additional samples were collected in the afternoon (1 PM and 5 PM) to obtain an accurate monitoring of the volatile fatty acid (VFA) composition. On day 5, samples for microbial and molecular analysis were collected just before starting continuous antibiotic administration, and 6 h thereafter (Figure 6.2).

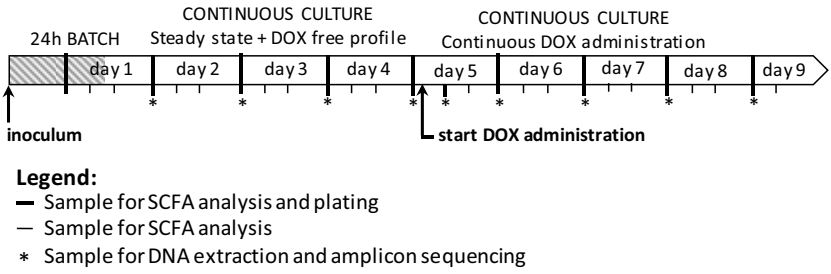


Figure 6.1 Chronology of a reactor run. Samples for plating and DNA extraction were collected at 9 AM. On day zero, the reactor was inoculated with a pooled cecal inoculum and operated as a batch culture during 24 hours. On day 1, the feed medium was prepared and the reactor changes to continuous culture. After sample collection on day 5, feed medium was supplemented with 1, 4 or 16 mg l^{-1} doxycycline hyclate. Six hours after starting continuous administration of doxycycline, an extra sample was collected for plating and DNA extraction.

6.2.2 Chemicals, growth medium and inoculum

The feed medium had a complex composition, mimicking the nutritional availability in the pig cecum. The chemicals were obtained from Sigma (Bornem, Belgium), unless stated otherwise. The feed medium consisted of (in g l^{-1}) starch from corn (5), casein from bovine milk (10), casein hydrolysate acid (0.5), soybean oil (1) (AD Delhaize, Belgium), anhydrous L-cystein hydrochloride (0.65), pectin from citrus peel (2.7), alphacel (13.8) (MP Biochemicals, Brussels, Belgium), mucin from porcine stomach type II (5), vitamin-mineral premix for pigs (2.35) (Vitamex, Drongen, Belgium), $KHPO_4$ (0.93) (Merck, Overijse, Belgium) and $Na_2HPO_4.12H_2O$ (1.12). The medium was acidified with 4 ml of 37% HCl to pH 2 and stored at 5°C in autoclaved 13 liter pyrex bottles under constant magnetic agitation. Beforehand, a zone of inhibition test confirmed that prolonged incubation at a pH of 2 had no negative influence on the activity of doxycycline (data not shown).

At the onset of each new experimental run, the reactors were inoculated with an identical and homogenous inoculum of cecal bacteria. To obtain a representative inoculum, ten organically grown pigs were selected from a farm that did not use antimicrobial therapy during growth. The cecal microbiota of these pigs as inoculum allowed to investigate the effect of antibiotics on an *ex vivo* microbial community that has not yet been into deliberate contact with antimicrobial compounds. The ceca of these pigs were removed at slaughter, tied up and individually stored in stomacher bags on ice for transport. In the lab, the cecal contents were poured out in a measuring cup and thoroughly stirred. The cecal content of 10 pigs were pooled to obtain enough inoculum for all technical repetitions of the reactor runs and for qPCR analysis

of *tet* genes. Subsequently, the pooled cecal contents were diluted with anaerobic phosphate buffer (8.8 g K_2HPO_4 + 6.8 g KH_2PO_4 + 1 g sodium thioglycolate in 1 liter dH_2O) to a 1:1 ratio, homogenized for 2 min in a stomacher and centrifuged for 3 min at 500 g. The supernatant was supplemented with 15% w/v glycerol and subsamples of 12 ml were stored at -80°C. Prior to inoculation, a subsample was thawed in a 37°C water bath.

6.2.3 Selective cultivation

The total number of CFUs and the number of tetracycline resistant CFUs of cultivable anaerobes, *Escherichia coli* and Enterobacteriaceae in the reactor were enumerated on agar plates. In these bioassays, we defined "resistance" as the ability of bacteria to proliferate in the presence of 10.5 mg doxycycline hyclate per liter agar. A 1 ml aliquot of each sample was used to make a 1:10 serial dilution series in 9 ml Maximum Recovery Diluent (Oxoid, Aalst, Belgium). 100 µl of each dilution was plated on Reinforced Clostridial Agar (RCA) (Oxoid), Violet Red Bile Glucose Agar (VRBGA) (Oxoid) and RAPID'E.coli2 Agar (Bio-Rad, Temse, Belgium) plates without DOX (total colony count) and with 10.5 mg l^{-1} filter-sterilized DOX (colony count of tetracycline resistant bacteria). The plates were prepared according to the manufacturer's instructions. RCA plates were anaerobically incubated at 37°C for three days, VRBGA plates were incubated for 24 h at 37°C and RAPID'E.coli2 plates at 44°C for 24 h before counting the colonies.

6.2.4 Fatty acid quantification

C2-C8 (including isoforms C4-C6) fatty acid analysis was performed according to Andersen et al. (2014) [375]. In short, liquid reactor samples were conditioned with sulfuric acid, sodium chloride and 2-methyl hexanoic acid as internal standard. Short chain fatty acids (SCFA) and branched chain fatty acids (BCFA) were extracted with diethyl ether and quantified with gas chromatography.

6.2.5 DNA extraction

DNA extractions were carried out as described in Chapter 2, section 2.2.2.

6.2.6 QPCR

QPCR quantification of bacterial 16S gene copies and the abundances of specific tetracycline resistance genes *tet*(Q), *tet*(W), *tet*(M), *tet*(O) and *tet*(B) was performed as described in Chapter 5, section 2.2.4.

6.2.7 Amplicon sequencing and processing of sequenced reads

Amplicon sequencing of the bacterial V3-V4 variable region of the 16S rRNA gene was done on 81 samples from nine reactor runs and on three replicate samples of the inoculum to determine the taxonomic profiles of the microbial communities. Library preparation and processing of the sequenced reads was carried out as described in Chapter 2, section 2.2.3 and Chapter 5, section 5.2.3. The raw sequence reads are available on the NCBI Sequence Read Archive under the project accession number PRJNA351773. The final OTU table was normalized using CSS [313] to account for variable library sizes, using the QIIME command "normalize_table.py" [260]. Processing the data resulted in an average library size of 57 199 reads per sample. Rarefaction analysis was done to ascertain that the library size of each sample was sufficient to analyze the bacterial community (Figure S6.1). Simpson diversity and observed richness were calculated with the Phyloseq package in R [264]. The OTU table was used to calculate relative abundances and summarize the table to family level, thus obtaining a table with the relative abundances of each bacterial family in samples collected from nine reactor runs and three replicate samples of the inoculum (i.e. cecal samples). The family level table was used to calculate pairwise Bray-Curtis dissimilarity indices and differences between communities were visualized with nonmetric multidimensional scaling (NMDS) using the Vegan package in R [263].

6.2.8 Statistical analysis

Significant changes in fatty acid concentrations, abundances of specific tetracycline resistance genes, total number of CFUs and number of resistant CFUs of *E. coli*, Enterobacteriaceae and cultivable anaerobes as a response to DOX administration were analyzed using a linear mixed effects model (R package lme4) [265] including "treatment" as fixed effect and "reactor run" as random effect, with Tukey adjustment for post-hoc testing. The short-term effect of DOX treatment on the microbial activity was analyzed by comparing the SCFA and BCFA data from two days prior to DOX administration with data from samples during the first two days of DOX administration. Significant changes in the number of CFUs of resistant species (plate

cultivation) and resistance gene abundance (qPCR assays) as result of DOX administration were tested in a similar way but using data from all sampling points. Significant results obtained with the linear mixed effects model were graphically verified: a pretreatment mean and a 95% confidence interval (CI95) were calculated for each parameter. Only parameter values that surpassed the CI95 boundaries during DOX administration, were accepted as a true significant response to DOX administration.

6.3 RESULTS

Data analysis of samples from reactor run 2 (1 mg l^{-1} DOX) revealed unexpected problems at the startup phase of this run. During the first two days of chemostat, the microbial community had a ten-fold lower concentration of main and branched chain fatty acids and metabarcoding revealed no *Bacteroidetes*. Concomitantly, the abundance of *tet*(Q) gene copies, mainly associated with *Prevotella* and *Bacteroides* [376,377], were much lower during the first two days as compared to other reactor runs. Because reactor run 2 deviated from all other runs, it was not considered representative and thus excluded from further analysis.

6.3.1 Microbial activity

Main SCFA and BCFA concentrations serve as markers for bacterial metabolic activity [378] and were used to assess steady-state after reactor startup and stabilization. Upon administration of 1, 4 and 16 mg l^{-1} DOX, average BCFA concentrations significantly decreased with 43% ($p < 0.001$), 36% ($p < 0.001$) and 28% ($p < 0.001$) in isovalerate concentration and 17% ($p < 0.001$), 20% ($p < 0.001$) and 14% ($p < 0.01$) in isobutyrate concentration, respectively (Figure 2). DOX administration also exerted an influence on the main SCFA concentrations. Propionate and butyrate concentrations significantly decreased with 18% ($p < 0.01$) and 33% ($p < 0.001$), respectively, during the administration of 4 mg l^{-1} DOX. Administration of 16 mg l^{-1} DOX resulted in a significant decrease of propionate and butyrate with 14% ($p < 0.05$) and 18% ($p < 0.001$), respectively, while 1 mg l^{-1} DOX administration resulted in an average 35% ($p < 0.001$) decrease of butyrate concentration. Acetate was the main SCFA of anaerobic fermentation but did not significantly decrease upon continuous administration of either one of the DOX concentrations (Figure 6.3).

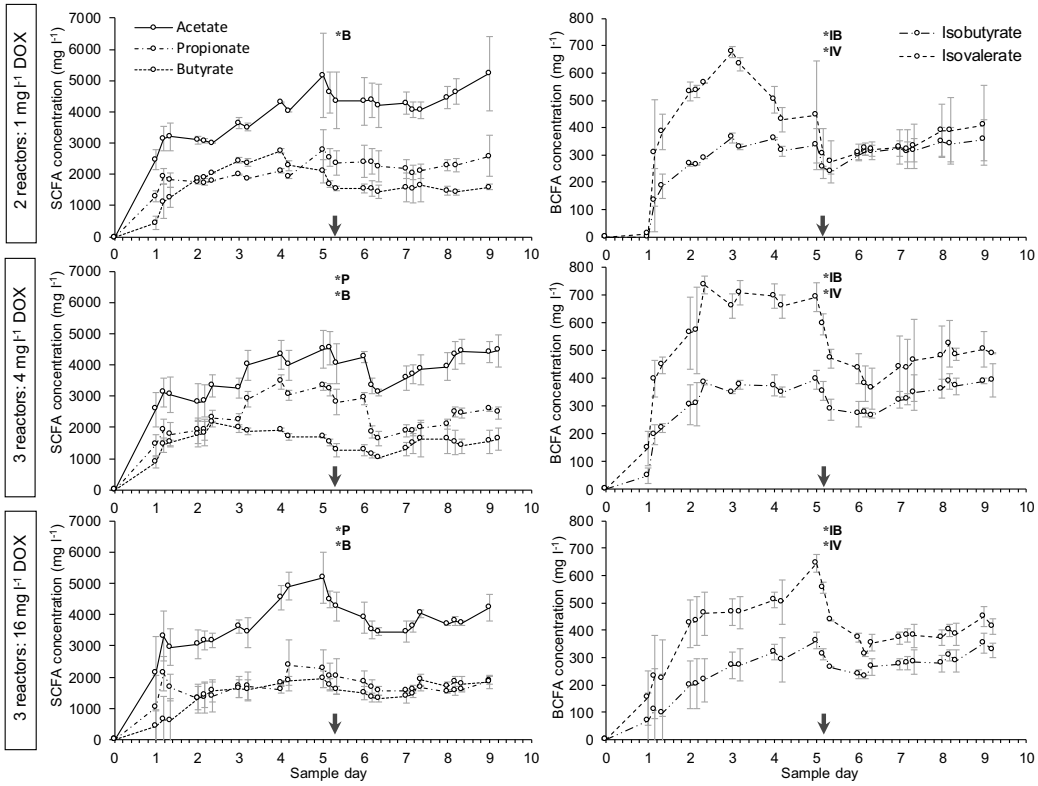


Figure 6.3 Mean concentrations (mg l^{-1}) of main short chain fatty acids (***left***): acetate, propionate and butyrate and branched chain fatty acids (**right**): isobutyrate and isovalerate. Means and standard deviations were calculated for 2-3 replicate reactor runs. Group 1 (**top**): reactor runs 1, 3 received 1 mg l^{-1} DOX. Group 2 (**middle**): reactor runs 4, 5, 6 received 4 mg l^{-1} DOX. Group 3 (**bottom**): reactor runs 7, 8, 9 received 16 mg l^{-1} DOX. The starting point of antibiotic administration is indicated by a vertical arrow. Significant short-term decrease of fatty acid concentrations, due to continuous administration of DOX, is indicated by a * (P = propionate, B = butyrate, IB = isobutyrate, IV = isovalerate).

6.3.2 Microbial community

The bacterial community structure of the *ex vivo* ecosystem was investigated with metabarcoding. Changes in richness (observed number of OTUs) and diversity (Shannon and Simpson index) measures could not be consistently attributed to DOX administration (Figure S6.2). On average, the main phyla of Bacteroidetes, Firmicutes and Proteobacteria represented 47% (± 6%), 39% (± 5%) and 6% (± 5%) of the reactor community before antibiotic treatment. The Enterobacteriaceae, a family within the Proteobacteria, represented only 0.9% (± 0.6%).

The Enterobacteriaceae were mainly represented by the genus *Escherichia-Shigella* which made up 0.7% (± 0.6%) of the entire microbial community. Taxonomic analysis of the bacterial communities identified a core set of eight families: the Prevotellaceae, Ruminococcaceae, Lachnospiraceae, Bacteroidaceae, Acidaminococcaceae, Erysipelotrichaceae, Enterobacteriaceae and Succinivibrionaceae. This dominant population was consistent between all reactor runs and represented 85% of the reads in all samples. The remaining families were prone to day-to-day fluctuation within each reactor run (Figure S6.3) and had variable relative abundances between reactor runs. To study differences between family level composition of the reactor communities, taking into account the relative abundances, samples were ordinated in a two-dimensional plot using NMDS analysis (Figure 6.4). Samples of the inoculum and samples collected on the second day of each reactor run cluster closely together. Samples of subsequent days are spread in the NMDS plot, where independent reactor runs display different positioning. Overall, there was no consistent increase or decrease of any taxonomic group as a result of 1, 4 or 16 mg l^{-1} DOX administration, which was observed in all of the replicate runs per DOX treatment.

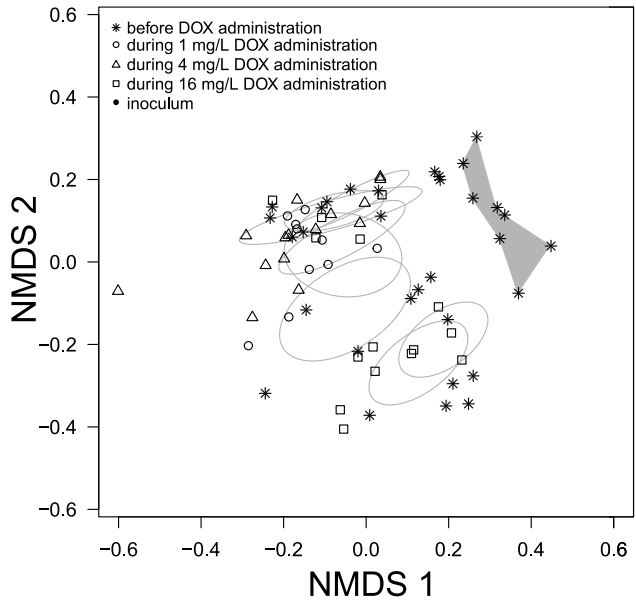


Figure 6.4 NMDS profile of pairwise community dissimilarity (Bray−Curtis) indices of family level abundance data of samples from eight reactor runs and three replicates of the inoculum. The symbols indicate the samples collected before DOX administration and during the continuous administration of 1, 4 or 16 mg l^{-1} DOX. The clustering of reactor samples collected on day 2 (when the system was transiting to steady-state) are indicated with spider graphics. 95% confidence ellipses were constructed for each reactor run.

6.3.3 Abundances of total and doxycycline resistant anaerobes, Enterobacteriaceae and *E. coli*

To determine the effect of 1, 4 and 16 mg l^{-1} DOX on the proliferation of resistant bacteria compared to the pretreatment period without DOX, reactor content was plated on agar for the enumeration of total and resistant CFUs of *Escherichia coli*, Enterobacteriaceae and cultivable anaerobes (Figure 6.5). During continuous administration of the lowest DOX concentration (1 mg l^{-1} DOX), only minor effects were observed compared to the control period of the reactors. The total *E. coli* count halved from 4.6×10^7 to 2.1×10^7 CFUs ($p < 0.001$) while the resistant *E. coli* count increased from 1.2×10^4 to 5.6×10^4 CFUs ($p < 0.001$). No significant effects were found for the enumerations of resistant and total Enterobacteriaceae and anaerobes (averages of 2 reactor runs). Administration of 4 mg l^{-1} or 16 mg l^{-1} DOX had a more pronounced effect on the proliferation of resistant bacteria. Resistant *E. coli* significantly increased from 1.1×10^2 to 1.1×10^5 CFUs ($p < 0.001$) and from 8.0×10^2 to 5.9×10^5 CFUs ($p < 0.001$), respectively, while resistant Enterobacteriaceae significantly increased from 4.4×10^5 to 2.5×10^6 CFUs ($p < 0.001$) and from 8.5×10^4 to 1.1×10^6 CFUs ($p < 0.001$), respectively (averages of 3 reactor runs). Total cultivable anaerobe counts were more variable between reactor runs. The resistant cultivable anaerobes increased from 7.9×10^7 to 8.1×10^8 CFUs ($p < 0.001$) and from 1.3×10^8 to 4.3×10^8 CFUs ($p < 0.01$), respectively (averages of 3 reactor runs).

6.3.4 Abundances of specific resistance genes

The quantification of tetracycline resistance genes and total 16S rRNA gene abundances was carried out to evaluate the influence of DOX administration on the abundance of specific resistance genes and on the total bacterial load in samples collected during each reactor run and in the cecal inoculum. The tetracycline resistance genes *tet*(Q), *tet*(O), *tet*(W), *tet*(M) and *tet*(B) were chosen because they are widespread and often detected with high abundances in agricultural environments [346,348,350,351] and occur in several bacterial groups of the gut microbiome [376]. The samples collected during the pretreatment period of the reactor runs had similar numbers of 16S genes and *tet* genes as detected in the inoculum samples (Figure 6.6.A): an average (± SD) bacterial load of 7.3 (± 2.5) × 10^8 16S gene copies ml^{-1}, 3.5 (± 2.0) × 10^6 *tet*(Q) genes ml^{-1}, 2.3 (± 8.6) × 10^6 *tet*(O) genes ml^{-1}, 4.5 (± 4.7) × 10^6 *tet*(W) genes ml^{-1}, 7.5 (± 9.4) × 10^4 *tet*(M) genes ml^{-1}, 1.4 (± 2.6) × 10^3 *tet*(B) genes ml^{-1}. The administration of the highest concentration (16 mg l^{-1} DOX) resulted in a near doubling of *tet*(W) concentrations,

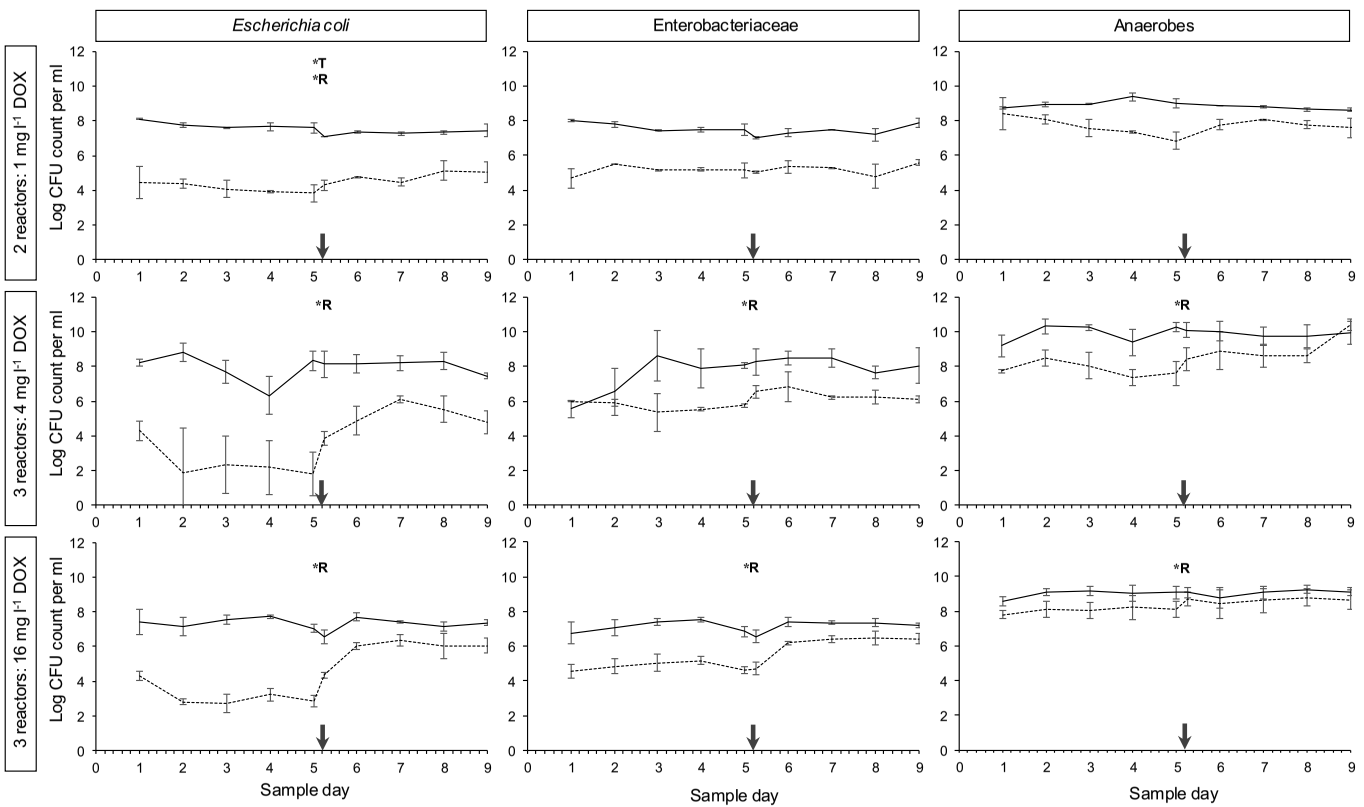


Figure 6.5 Mean enumerations of total (**full line**) and doxycycline resistant (**dashed line**) *E. coli*, Enterobacteriaceae and cultivable Clostridia given in $\log_{10}$ CFU counts per ml reactor content. Means and standard deviations were calculated for 2-3 replicate reactor runs. Group 1 (**top**): reactor runs 1, 3 received 1 mg l^{-1} DOX. Group 2 (**middle**): reactor runs 4, 5, 6 received 4 mg l^{-1} DOX. Group 3 (**bottom**): reactor runs 7, 8, 9 received 16 mg l^{-1} DOX. The starting point of antibiotic administration is indicated by an arrow. Significant increases/decreases in colony count per ml (T = total colony count, R = doxycycline resistant colony count on agar containing 10.5 mg l^{-1} DOX) due to continuous administration of doxycycline, is indicated with a *.

which significantly increased with 3.7×10^6 gene copies ml^{-1} ($p < 0.01$). The concentrations of *tet*(M) significantly increased with 1.7×10^5 gene copies ml^{-1} ($p < 0.01$) and *tet*(B) gained 3.6×10^3 gene copies ml^{-1} ($p < 0.001$), representing a three -and tenfold increase, respectively. No significant changes were observed for *tet*(Q) and *tet*(O). The administration of lower concentrations (1 or 4 mg l^{-1} DOX) did not induce a significant increase of *tet*(Q), *tet*(O), *tet*(W), *tet*(M) (data not shown) or *tet*(B) (Figure 6.6.B).

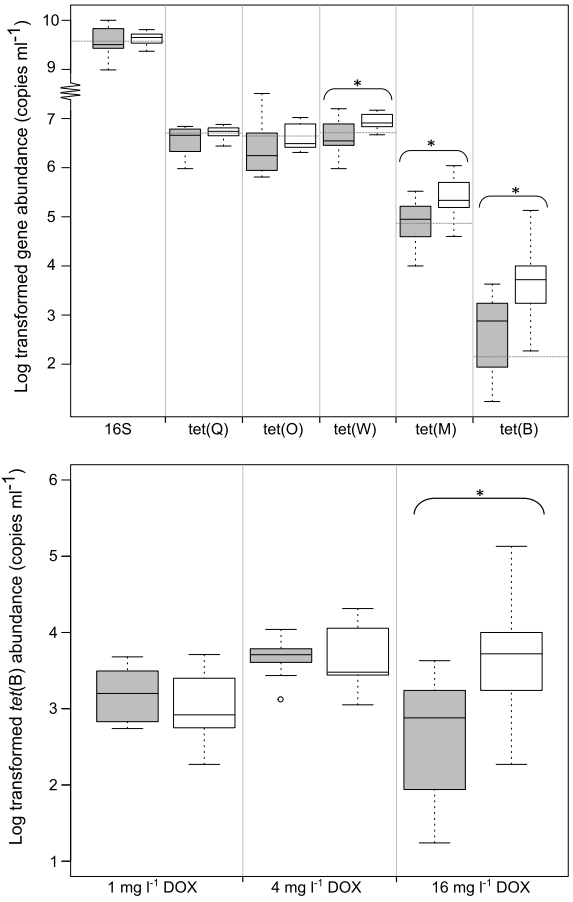


Figure 6.6 [A] Box blot of gene abundances before (**grey**, n=12) and during (**white**, n=15) 16 mg l^{-1} DOX treatment in three reactor runs. The 16S rRNA and specific tetracycline resistance genes *tet*(W), *tet*(O), *tet*(Q), *tet*(M) and *tet*(B) are expressed as $\log_{10}$ transformed gene copies per ml reactor content. The dashed lines indicate the abundance of the respective gene in 1 ml inoculum fluid. **[B]** Box plot of *tet*(B) gene abundance before (**grey**, n=12) and during (**white**, n=15) administration of 1 mg l^{-1} DOX (reactor runs 1, 3), 4 mg l^{-1} DOX (reactor runs 4, 5, 6) and 16 mg l^{-1} DOX (reactor runs 7, 8, 9). *Tet*(B) Significant differences in gene abundance before and during doxycycline administration are indicated with *.

6.3.5 Comparing the *in vivo* and *in vitro* microbial ecosystem

The reactor vessels were inoculated with pooled cecal content, harvested from 10 organically grown pigs, comprising 997 ± 23 OTUs and a Shannon diversity of 4.65 ± 0.03 (three replicate samples). The microbial communities in the bioreactors counted on average 217 ± 24 OTUs and had a Shannon diversity of 3.52 ± 0.12 (n = 45). According to the Venn diagram (Figure 6.7) constructed after retaining only those OTUs with a relative abundance of 0.01% in at least one of the samples, the majority of the OTUs (i.e. 464 OTUs) were shared between the reactor communities and the *in vivo* communities. Taking into mind that the a reactor vessel, at a given time, houses a community of about 217 OTUs, the Venn diagram suggest that the reactor conditions could select for a range of different species which also made up the dominant members in the *in vivo* cecal community as they cumulatively accounted for 81% of the reads, whereas the OTUs not included in the reactor microbiomes only represented 19% of the reads in the cecal samples.

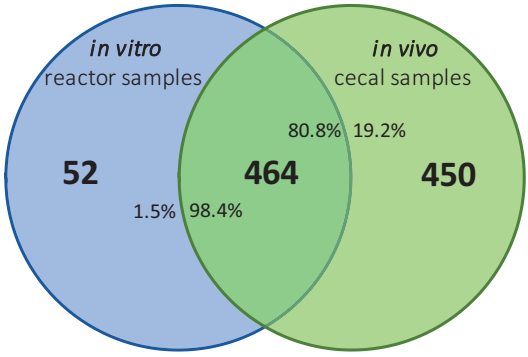


Figure 6.7 Venn diagram describing the average OUT distribution across *in vitro* samples collected from the bioreactors and the cecal inoculum.

The beta-diversity between the reactor samples and the inoculum is investigated by using the phylogenetic distances between OTUs. Differences between closely related species are given lower weights on the assumption that related species have similar genetic capacities. Weighted and unweighted UniFrac distance metrics, which incorporate the phylogenic relatedness between community members, were calculated for each sample and ordinated with principle coordinates analysis (PCoA) (Figure 6.8). PCoA of unweighted UniFrac distances (using sequence jackknifing), which only considers the presence or absence of OTUs, indicates

substantial compositional differences between the *in vitro* bacterial communities and the inoculum as a result of the inequality in richness between both sample types. However, in the PCoA of the weighted UniFrac distances, the cecal inoculum are positioned amongst the *in vitro* samples, indicating a very comparable community composition and structure of the *in vivo* and *in vitro* samples, despite their differences in richness and diversity. The variation between reactor samples (observed as the spread positioning in the PCoA plot) suggest an influence of reactor run and sampling time on the microbial community structure. Apart from the microbial community, also the SCFA profiles were compared between the *in vitro* simulation of the pig's cecum and the *in vivo* samples collected from the ceca. The cecal samples had a total concentration of 9-10 mg SCFAs per g of intestinal content, represented primarily by acetate (54%), propionate (27%) and butyrate (16%). Similarly, the reactor samples contained an average of 8.7 ± 1.1 mg SCFAs per ml, with steady-state distributions of acetate (48 ± 4%), propionate (25 ± 4%) and butyrate (19 ± 3%).

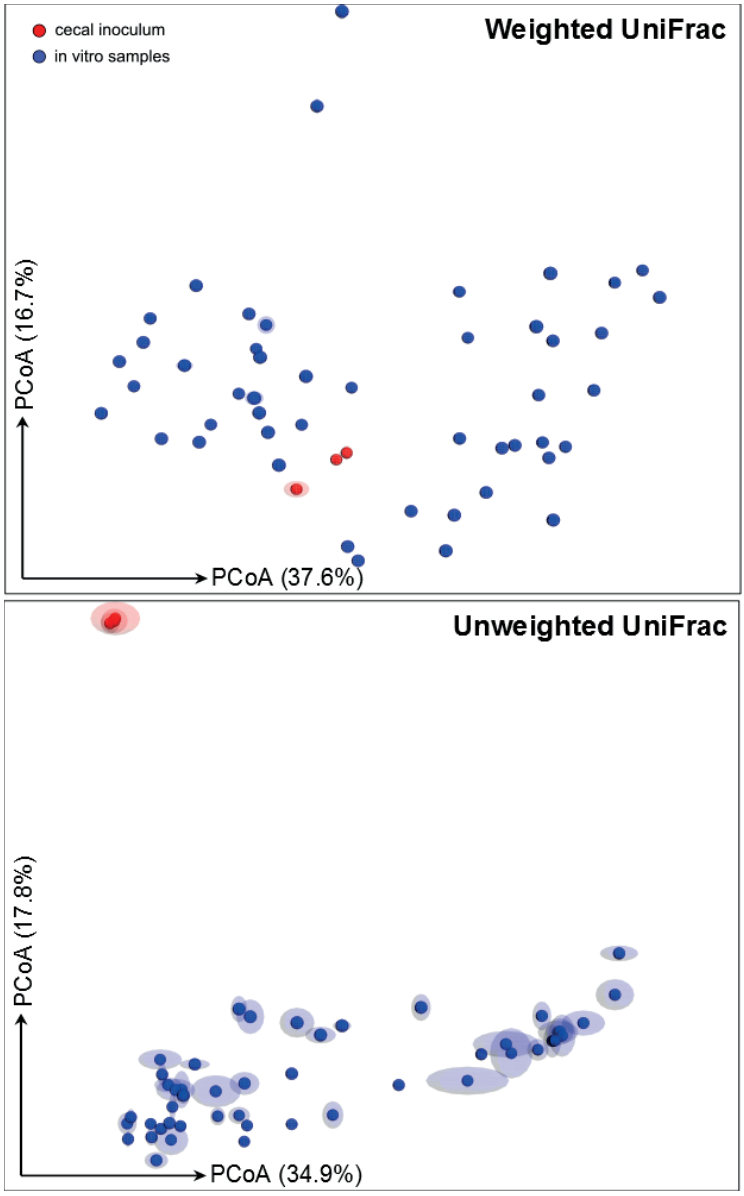


Figure 6.8 ordination of weighted (**top**) and unweighted (bottom) UniFrac distances using principle coordinates analysis. Jackknife resampling was carried out and the spherical points indicate the averages and the ellipses represent the variance between repeats.

6.4 DISCUSSION

6.4.1 Evaluation of the reactor model as simulation of the cecal microbial ecosystem

The *in vitro* model was designed to mimic the chemical and physical conditions of the cecal microbial ecosystem. At the onset of each run, the vessels were inoculated with a rich inoculum. During an initial 24h batch incubation, the inoculated viable bacteria could proliferate in the reactor system. Subsequently, the reactors were operated as chemostat under strictly regulated conditions: a defined feed medium, pH of 6.5, temperature of 37°C and a residence time of 4.6h. These strict ambient conditions imposed harsh selective criteria on the inoculated bacteria resulting in an *in vitro* microbiome with a four-fold lower richness and a lower diversity as compared to its *in vivo* counterpart. However, despite the observed differences in richness and diversity, the ordination of weighted and unweighted UniFrac distances suggest that the *in vitro* microbiome resembled the cecal microbial community both in terms of abundant bacterial taxa and their proportions, as well as their functionality (as determined by VFA productions and proportions). The *in vitro* reactor system can therefore be considered as an adequate model to simulate the pig's cecal microbial ecosystem. The formation of the microbial communities during *in vitro* fermentation was simultaneously governed by deterministic and stochastic processes. Habitat specialization plays a pivotal role in assembling the *in vitro* microbiome as the ambient conditions in the reactor select for specific taxa able to cope with the controlled environment and nutritive availabilities. However, the complex cecal microbiome from which the inoculum was made, is characterized by functional redundancy (i.e. multiple taxa able to occupy the same niche) and the presence of generalist species (i.e. species that can occupy multiple niches), allowing stochastic forces to influence the microbial assembly as coincidence and random occurrences (fluctuation in pH, temperature, nutritive availabilities, etc.) determined which taxa could proliferate in the reactors.

6.4.2 The influence of carry-over concentrations of DOX on the cecal microbial ecosystem

Carry-over of antimicrobial compounds from medicated feed to non-medicated feed results in the presence of subtherapeutic concentrations of antimicrobials in the non-medicated feed. When pigs consume these cross-contaminated feed, such an antimicrobial compound can accumulate in the compartments of the intestinal tract where it might exert an influence on the microbial community and activity and impose a selection pressure for resistant bacterial species

or strains. The objective of this study was to investigate the effects of such subtherapeutic doses of DOX. 1 and 4 mg l^{-1} DOX correspond to the *in vivo* intestinal concentration range upon feeding with a compound feed containing 3% of the maximum recommended dose, which was determined in a previous study [173]. These subtherapeutic concentrations, and a positive control concentration of 16 mg l^{-1} DOX, were administered to the complex microbial community of an *ex vivo* continuous fermentation model, mimicking the chemical and physical characteristics of the pig cecum. The *ex vivo* simulation of the pig cecum allows to study the influence of an antibiotic on a microbial community under strictly controlled conditions, which increases repeatability and eases sample collection. The bioreactors were inoculated with the cecal content of organically grown pigs (who did not receive antibiotic treatments during growth) to investigate the effect of DOX on a gut microbial community that has not yet been into contact with antimicrobial compounds. With this setup, we argued that the effect of first contact of subtherapeutic (1 or 4 mg l^{-1}) concentrations of DOX on the intestinal microbiome of pigs could be investigated.

Doxycycline inhibits protein synthesis of susceptible bacteria by binding on the 30S subunit of the ribosome and preventing the attachment of aminoacyl-tRNA. Consequently, doxycycline is a broad-spectrum antibiotic, exerting a bacteriostatic effect. The fatty acid profiles suggest that even the administration of low concentrations of DOX (1 mg l^{-1}) can cause inhibition of protein synthesis of sensitive bacteria, resulting in reduced metabolic activity and lower production of metabolic end products. The steep reductions of BCFA concentrations, as compared to SCFA, are likely due to the tenfold lower concentrations of BCFAs causing a more pronounced reduction. Another possible explanation might be the variable effect of doxycycline on the solid adherent and the liquid environments of the ecosystem. The presence of solid adherent bacteria is evidenced by the detection of known [379] cellulolytic genera such as *Ruminococcus* (1.8 ± 3.3%) and *Fibrobacter* (2.3 ± 3.7% of the reads) using metabarcoding. Microbial attachment and the development of biofilms on the surface of particles (mainly provided by insoluble alphacel which makes up 37% of the feed medium) is the driving factor behind carbohydrate fermentation [131] which accounts for a large fraction of the acetate, propionate and butyrate concentrations [380]. Presumably solid adherent bacteria are, to a certain extent, protected against doxycycline (this hypothesis is further explored in Supporting Information, S4). On the other hand, proteolytic activity is primarily attributed to free-living bacteria, proliferating on soluble nitrogen sources in the feed medium and generating a complex mixture of metabolic end-products, including SCFAs (acetate, butyrate and propionate) and

BCFAs (isobutyrate and isovalerate) [239]. These free-living bacteria are more susceptible to antibiotics [381], which could also have contributed to the steep decrease of BCFA production following DOX administration.

In contrast to the effects of DOX on the metabolic activity, alpha diversity calculations and ordination of the samples based on OTU abundance profiles suggest there was no influence of DOX administration on the microbial community structure. In line with these results, no taxonomic group was found to significantly increase or decrease during continuous DOX administration. Similarly, Holman and Chénier (2013) demonstrated that the microbial diversity and community structure of fecal samples were not affected by administering a subtherapeutic dose of 5.5 mg chlortetracycline per kg feed to weaning pigs [382]. Presumably, selection pressure imposed by doxycycline will cause resistant bacteria to proliferate and replace sensitive species within the major taxonomic groups. In addition, adherent bacteria and bacterial aggregates could contribute to the stability of the community during antibiotic stress. Therefore, our findings suggest that doxycycline administration only exerts a minor influence on the bacterial community composition.

Plate cultivation on agar with DOX indicated already a presence of around 10^2-10^4 tetracycline resistant *E. coli* per ml (representing between 0.0001% and 0.02% of the total enumerated *E. coli* counts) and around 10^7-10^8 tetracycline resistant cultivable anaerobes per ml (representing between 0.7% and 13% of the total enumerated anaerobes) in the reactors during the pretreatment period. This was remarkable as there was no selective pressure imposed by antibiotics during the pretreatment period nor during the growth of the organically grown pigs who's cecal content were used as inoculum of the bioreactors. In comparison, 40.4% of *E. coli* isolated from fecal samples from organically grown Tibetan pigs (with a complete absence of antibiotic use either therapeutically or for growth promotion) displayed resistance against tetracycline [370]. It would seem that tetracycline resistant species are an inherent part of the microbial communities in the pig's GIT. Despite the high occurrence of tetracycline resistance in the intestinal microbial ecosystem of pigs (even in organically grown pigs), selective plate cultivation in our experiments provided strong and consistent evidence of the influence of DOX on the enrichment of tetracycline resistant species in the complex and dynamic microbial communities of the reactor system. Notably, when focusing on a single species such as *Escherichia. coli*, a generally recognized indicator organism for tracking microbial resistance [383] and omnipresent in the community of each reactor run, we observed a profound effect of DOX administration on the enrichment of resistant CFUs. In contrast, the effects of DOX

administration were smaller when larger groups were enumerated on less specific agar plates, such as the family of Enterobacteriaceae on VRBGA and cultivable anaerobes (mostly of the class of Clostridia) on RCA, as these plates support the growth of multiple species and each species can respond differently to the effects of DOX. Nevertheless, plate cultivation suggest that subtherapeutic concentrations of DOX can cause significant enrichment of tetracycline resistant species, especially species that form a potential threat to human and animal health, for which *E. coli* is an indicator.

In accordance with the high prevalence of resistant CFUs during the pretreatment period of the reactor runs, qPCR assays also indicated high concentrations of specific *tet* genes during the pretreatment period: around 10^9 gene copies per ml of eubacterial 16S rDNA were detected and around 10^6 gene copies per ml of *tet*(Q), *tet*(O) and *tet*(W), 10^5 gene copies per ml of *tet*(M) and 10^3 gene copies per ml of *tet*(B). Furthermore, the bioreactors contained similar levels of *tet*(Q), *tet*(O), *tet*(W) and *tet*(M) during the pretreatment period of the reactors as detected in the inoculum prepared from the pooled cecal content of organically grown pigs. These results suggest that tetracycline resistance is omnipresent in a gut environment and *tet* gene levels remain unaffected by the rapid transit time in the reactors, selecting for rapidly growing bacteria in the cecum. Long-term persistence and ubiquity of tetracycline resistance genes in the absence of antibiotic use was also previously suggested [197,384]. However, in contrast to plate cultivation, the concentrations of *tet* genes did not increase upon DOX administration of 1 and 4 mg l^{-1} and only 16 mg l^{-1} caused statistically significant increases of *tet*(W), *tet*(M) and *tet*(B). These findings indicate a discrepancy between classic plate cultivation of viable bacteria and the molecular quantification of genes. This could be attributed to the limited number of *tet* genes investigated in this study. Five *tet* genes were selected and quantified because of their frequent occurrence in agricultural environments with antibiotic use [348,350,351]. *Tet*(Q), *tet*(O), *tet*(W) and *tet*(M) encode ribosomal protection proteins and are found in both Gram-positive and Gram-negative bacteria. *Tet*(B) encodes a widely distributed Gram-negative tetracycline efflux pump [211] but is mainly associated with species of the *Proteobacteria* [376,377]. However, there is a large number of known (and yet unknown) *tet* genes that were not included in this study. The effect of DOX administration on the abundance of a single *tet* gene might be limited because numerous other resistance genes and/or mutations may contribute to resistance development, thus spreading the effect across a large number of genes. It is therefore possible that DOX administration only induces a limited increase in the abundance of a large number of *tet* genes.

Plate cultivation and qPCR are commonly used techniques in research on antimicrobial resistance, but our results emphasize the shortcomings of these methods. Agar plate cultivation enumerates total and resistant cultivable bacteria, but lacks in information about the taxonomy and the antimicrobial resistance determinants they possess. Furthermore, only a small fraction of the community is cultivable. On the other hand, each qPCR assay is specific for a resistance gene or a group of genes, thus a researcher must make a reasoned selection of which genes to study, with the risk of overlooking other important contributors. In addition, qPCR only provides quantitative information of gene levels, but not about the host association. Developments in the field of metagenomics can address these shortcomings. Hi-C sequencing, based on DNA crosslinking in living cells prior to NGS library preparation, is able to reliably associate plasmids with each other and with the chromosomal DNA of the host cell [385]. A recently described technique called epicPCR isolates single bacterial cells in emulsion beads and uses fusion PCR to physically link specific functional and phylogenetic genes prior to amplicon sequencing [386]. In the near future, these novel techniques could be used to correlate taxonomic classification and resistance mechanisms of the species in the community.

6.5 CONCLUSION

The *in vivo* simulation proved an appropriate model of the microbial ecosystem in the pig's cecum. Using this model, we investigated the influence of a positive control concentration (i.e. 16 mg l^{-1} DOX) and subtherapeutic doxycycline concentrations, as a consequence of cross-contamination in the feed, and a on the microbial ecosystem of the pig cecum simulated with an *ex vivo* bioreactor model. When cross-contamination results in a concentration of 3% or more of the therapeutic dose, there is a significant effect on the enrichment of resistant bacteria and specifically resistant *E. coli*. As specific *tet* genes were already abundant in the ceca of organically grown pigs used as inoculum and in the pretreatment phase of each reactor run, only the highest DOX concentration tested led to a small increase in abundance of the investigated *tet* genes. On the other hand, the microbial activity, indicated by the fatty acid concentration, decreased significantly for each DOX concentration tested, whereas the taxonomic profile of the simulated bacterial cecal community was not influenced by DOX administration.

6.6 SUPPLEMENTARY DATA

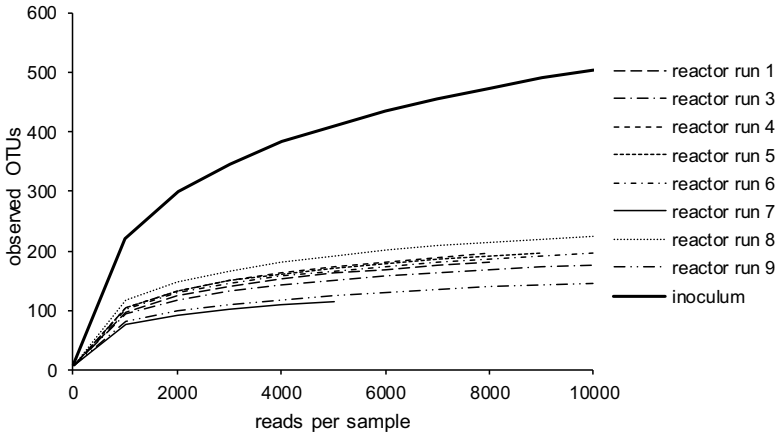


Figure S6.1 Rarefaction curves of the microbial communities of each reactor run and in the inoculum. Analysis was done using an upper rarefaction depth of 10.000 reads.

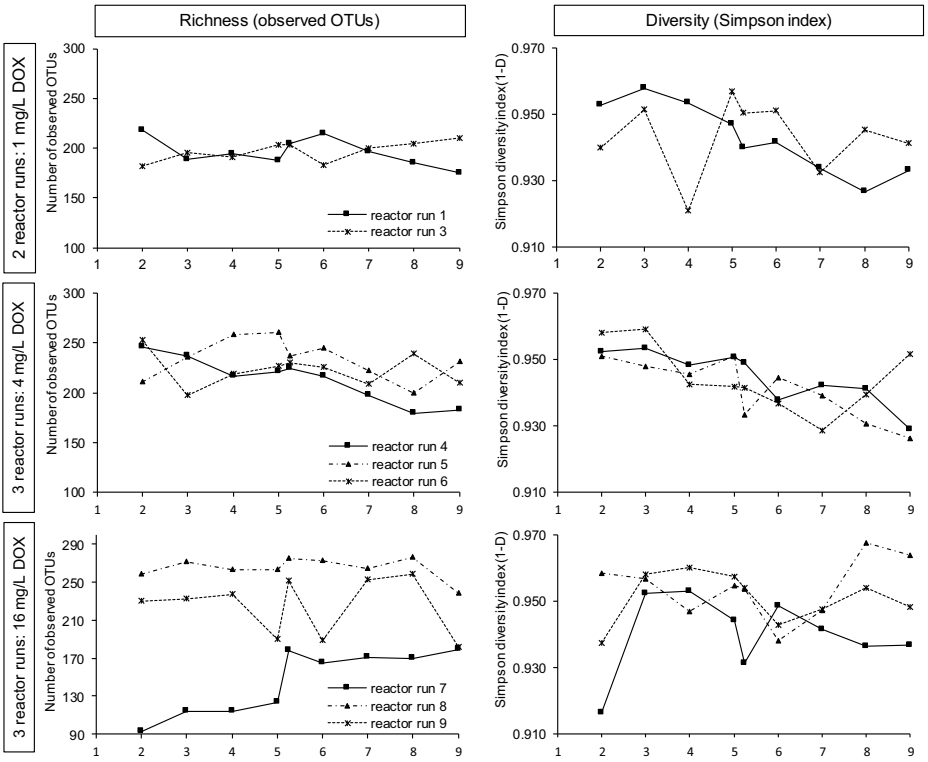


Figure S6.2 Richness measures (observed number of OTUs) and Simpson diversity indices of each reactor run. Group 1 (**top**): reactor runs 1, 3 received 1 mg l^{-1} DOX. Group 2 (**middle**) reactor runs 4, 5, 6 received 4 mg l^{-1} DOX. Group 3 (**bottom**) reactor runs 7, 8, 9 received 16 mg l^{-1} DOX.

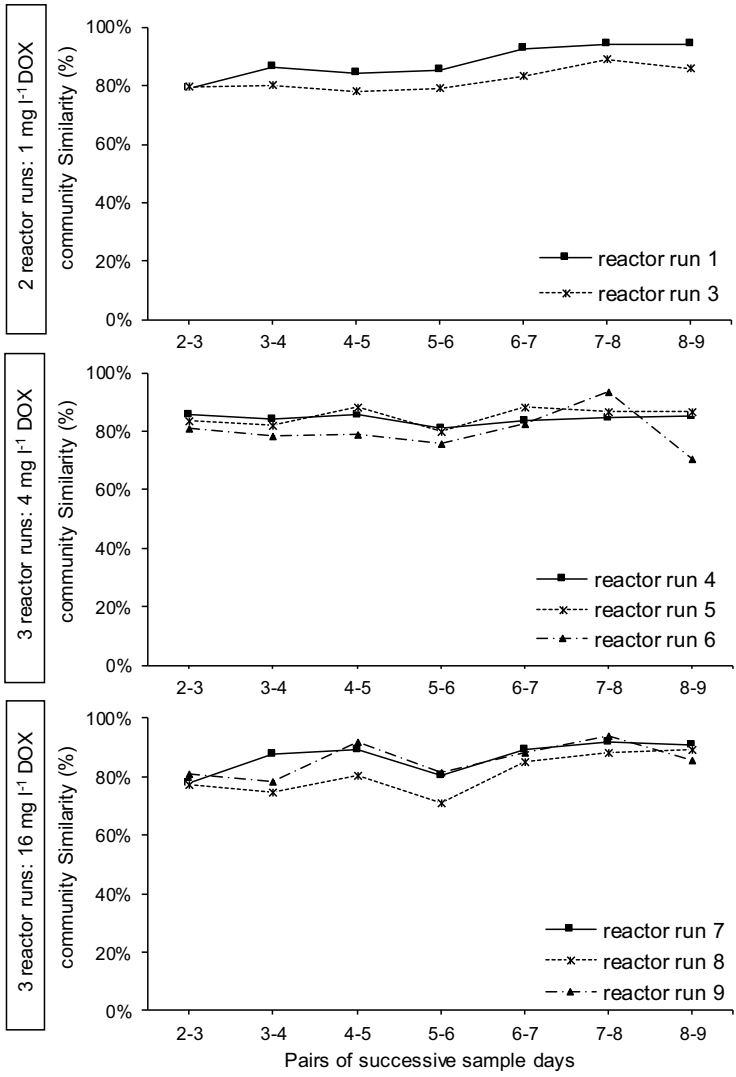


Figure S3 Similarity between communities of successive days of a reactor run was evaluated using moving window analysis. Bray-Curtis dissimilarities (1-BC) were converted to similarity values and plotted against the time interval.

S4

Mechanisms of how bacteria adherent to insoluble feed particle might be protected against the negative influences of antibiotics

Bacteria often prefer a surface-bound lifestyle to a planktonic existence. Bacterial adhesion provides protection against external factors (ex. predation by protozoa, toxic components, environmental fluctuations), encourages symbiotic relations and often allows direct access to nutrients. In aquatic ecosystems, surface associated bacteria vastly outnumber planktonic bacteria.[387] Especially microbial cellulose digestion is exclusively performed by adherent cellulolytic bacteria.[53] Dominant fiber adherent populations establish within the first hour of fermentation[245,388] and will proliferate, encapsulate themselves and develop into a biofilm.

In our reactor setup, either one of two possibilities could be applicable: (1) The solid adherent bacteria developed biofilms, which would have provided protection against the negative influences of antibiotics[389] or (2) the rapid transit time of the chemostat did not permit biofilm formation. If this was the case, we hypothesize that single-layered attached bacteria also enjoy a form of protection against antibiotics due of their adhesion to the particle. Fick's first law of diffusion postulates that a solute (i.e. doxycycline) will move from a region with high concentration (i.e. the homogenous liquid environment) to a region of low concentration (i.e. the particle surface) across a decreasing concentration gradient. Thus the particle was surrounded by an external liquid film layer that poses restrictions on mass transfer. As a result, the immediate vicinity of the particle surface had a lower antibiotic concentration.

Chapter 7

General discussion and perspectives

CHAPTER 7 GENERAL DISCUSSION AND PERSPECTIVES

7.1 TECHNOLOGIES TO STUDY MICROBIAL COMMUNITIES

7.1.1 The advantages and challenges of metabarcoding

Next generation sequencing (NGS), also known as high throughput sequencing (HTS), is a catch-all term to describe a number of modern technologies to sequence nucleotides more rapidly and cheaper than Sanger sequencing. These novel techniques are capable of simultaneous sequencing millions of reads, offering unparalleled data generation and revolutionizing the study of complex biological systems. Culture independent community fingerprinting techniques to profile the microbial diversity and population structure, such as DGGE [390], ARISA [391] and RFLP [392], gradually become outdated and are slowly replaced by metabarcoding. The power of this NGS technique lies in the capability of massive parallel multiplex DNA sequencing. After DNA extraction, a specific DNA sequence is amplified (amplicon PCR) and provided with sample-specific indices (index PCR). This allows simultaneous sequencing of multiple (usually 96-120) samples. Reads of each sample can be differentiated by sample-specific "barcode" sequences at the terminal end of each sequenced read. Although metabarcoding scores better on a large number of points, it still has several technical drawbacks in common with molecular fingerprinting. The choice of sample collection procedure, DNA extraction procedure, primers (determining which genomic region is amplified) and sequencing platform will influence to a large extent the output data. After obtaining the raw sequenced data, the number of options becomes even broader. For each step in the data processing pipeline, going from read preparation (assemble paired-end reads, quality filter/trimming), dereplication, OTU clustering, chimera removal to OTU identification, quite a few commercial or free-of-charge packages are available to choose from. Each of these packages or programs operate uniquely because of their distinct algorithms and features, that determines what the end-result will look like. For example, the stringent conditions of quality filtering will determine the number of retained reads and the choice of the database used for sequence annotation will determine the final community composition at different taxonomic levels. Luckily, there are only a few complete packages that provide pipelines for all the above mentioned processing steps during microbiome analysis. QIIME [260], UPARSE [257] (both based on USEARCH) and mothur [393] are amongst the most popular, often in combination

with the SILVA [394], Greengenes [261] or RDP database [395] (all containing chimera-checked and quality-controlled 16S rRNA genes).

During OTU clustering, the 16S rRNA gene sequences are usually clustered together at a 97% similarity threshold. From each OTU (a cluster of reads with >97% similarity), a representative sequence is selected for subsequent taxonomic annotation. It is therefore assumed that each OTU approximately corresponds to a single unique species. This assumption may fail as different species may have 16S rRNA genes with more than 97% similarity, causing one OTU to represent multiple species, or a single species may have paralogues of the 16S rRNA gene that are less than 97% similar, causing a single species to become represented by two OTUs . Obviously, there has been a lot of criticism on using 97% percent sequence similarity to define OTUs and many authors have described OTU clustering at 98% [396] or 99% [397], though these are still bound by the same drawbacks. Nonetheless, percentage similarity clustering at 97% is the most frequently used due to the computational benefit and the established habit of uniformity with other studies and protocols. Alternatively, dissimilarity metrics can also be used to quantify evolutionary distances between pairs of sequences and form a more substantiated option to generate OTUs. Nguyen et al. (2016) examined the accuracy of three of these metrics: pairwise alignment sequence dissimilarity, MSA-based sequence dissimilarity and phylogenetic branch length distance. Their results suggest that we need to move beyond the simplistic clustering techniques and thrive towards methods that classify every read instead of looking at representative sequences for taxonomic identification [398].

Considering the wide range of options you are faced with as a researcher in the course of your experiment, sample collection, DNA extraction, library preparation and data processing can have a large impact on how you eventually perceive the community profiles. It is therefore difficult to compare results between different studies and the responsibility of researchers and journals to provide complete information of used programs and protocols. Fortunately, many quality journals already require that authors make their raw sequencing data publically accessible, so that you can compare your data with published data using your own pipelines. Nevertheless it could be beneficial to strive for more uniformity in DNA extraction, library preparation and data processing procedures amongst different research labs studying similar ecosystems. The use of uniform standard operating procedures (SOPs) would enhance data sharing and comparing while also guaranteeing the quality and integrity of results. The importance of standardized SOPs across different research groups inspired the International

Human Microbiome Standards (IHMS) project. Twenty-three contributing institutes from twelve countries put their heads together to develop and implement SOPs for sample collection and processing, sequencing genes and genomes and organizing and analyzing data in order to make results optimally comparable [399]. Other scientific communities are also making an effort to share strategies for analysis and data interpretation. In 2014, the European Food Safety Authority (EFSA) organized a meeting about the use of WGS to identify foodborne pathogens and to harmonize approaches for data analysis [400]. These projects and meetings, focusing on sharing expertise and technologies, are ideal platforms to promote procedures and technology that have been generally agreed upon by the scientific community. Though it should be kept in mind that imposed uniformity should not come at the risk of reduced investments in optimization of existing protocols and pipelines.

Due to the lack of standardized procedures for investigating the microbial communities of intestinal ecosystems by metabarcoding, the first months of this PhD were spent by searching frequently described methodologies in literature and evaluating them in the lab. Sampling procedures to collect rumen fluid, fibers and epithelium were optimized. Three DNA extraction procedures were compared for DNA quantity, quality and metabarcoding results. Two primer pairs were compared to specifically investigate the rumen methanogen community using metabarcoding. Based on these preliminary experiments, (data not shown) protocols were retained that were used in all the experiments during this PhD. DNA extraction was performed using the Repeated Bead Beating and Column protocol (RBB+C) as described by Yu and Morrison (2004) [250]. Library preparation for sequencing was done with the primer pair proposed by Illumina and first described by Klindworth et al. (2013) [268] for the bacterial community, and using the primer pair of Kittelman et al. (2013) [253] for the methanogen community.

7.2 STUDYING FACTORS THAT DETERMINE THE RUMEN MICROBIAL COMMUNITY

7.2.1 What determines the rumen microbial community composition?

Ruminant livestock production constitutes a major component of the global agricultural economy and has therefore been the focus of many scientific studies aiming to improve the zootechnical performance like growth and production metrics or to reduce environmental impact, of which enteric methane emissions by cattle is an important aspect. Methane is

produced in the rumen of cattle as a byproduct of anaerobic fermentation. Other parameters such as milk yield, feed efficiency and growth are also directly or indirectly related to the functioning of the rumen, and thus related to the rumen microbial community. The prokaryotic community, which is the main component of the rumen microbial biomass, is the driving force behind feed digestion and fermentation. Investigating the rumen prokaryote community can thus provide added value to any study focusing on methane mitigation, improving feed efficiency or increasing milk yield as these factors are interdependent. Large *in vivo* studies have indicated large between-animal variations in milk yield [401], methane emissions [112,115,116,243] and feed efficiency [402,403], in spite of efforts to standardize influencing factors. These variations may reflect different microbial communities amongst animals, even under conditions of equal nutrition, environment and physiological stage. This PhD work investigated those factors that influence the microbial community composition and activity and through that, also influence related parameters such as milk yield, feed efficiency, growth and methane production. To this end, experiments were designed to investigate the difference between microbial community compositions of rumen environments (Chapter 2), to determine the influence of host (Chapter 3) and the influence of breed (Chapter 4). The influence of the diet type, which is generally accepted as the main driver of methane emissions [140] and the main determinant of the rumen microbial structure [333], the milk yield and animal growth, was not a focus in this PhD as this has been extensively investigated in the past [97,140,404].

An experimental design, aimed at investigating the rumen microbial community and/or activity, will stand or fall by sample collection, as this has to take into account the diurnal fluctuations and the differences between environments. To investigate the differences between the microbial composition of the three main rumen fractions, i.e. the liquid, solid adherent and epimural fraction, samples were collected from these environments from four cannulated cows, under equal dietary, physiological and ambient conditions (Chapter 2). The three environments differed from one another in terms of richness and diversity and taxonomic composition of both the methanogen and bacterial communities. Further, this experiment showed that straining the rumen liquid to remove fibers is not necessary because crude rumen liquid samples (collected with a probe and vacuum pump) provide a similar picture of the rumen microbial structure of the liquid fraction. On the other hand, the labor-intensive protocol to elute solid-adherent bacteria from fibers fails to provide a complete picture of the solid-adherent bacteria, presumably because tightly attached bacteria are inadequately eluted and thus underrepresented in the sample. A better method for analyzing the solid-adherent fraction is simply performing

DNA extraction directly on strained and washed rumen solids. This procedure is less labor intensive, less biased by manual operations, independent of elution and represents more accurately the solid-adherent microbes. The collection of epimural samples by scarping the rumen wall with a curette did not generate comparable results amongst the tested animals, as these samples were often "contaminated" with residual fibers and liquid, despite that the rumen was emptied and rinsed. Epimural sample collection forms a big challenge as they can only be collected from the (emptied) rumen of slaughtered or cannulated cattle by scraping the rumen wall or excising papillae. Nevertheless, the epithelial samples indicated a bacterial and methanogen community population that stands apart from the communities in the liquid and solid environments, in terms of composition and function.

The liquid and solid environments are interdependent. Mature biofilms disperse bacteria to the fluid, from where they can migrate to newly ingested feed to generate biofilms. Following this reasoning, crude rumen liquid samples, including the planktonic species (consuming soluble nutrients) and species dispersed from biofilms and solid-adherent colonies, best represents the entire rumen microbial community. Nevertheless, 80-90% of the bacteria are associated with particulate matter, and these microbial communities thus represent the most important role in rumen digestion and methane production [126]. Therefore, specific sampling of the solid-adherent community (rather than the rumen fluid environment by CRL samples) could provide a better understanding of those bacteria responsible for the majority of the microbial fermentation activity.

The origin of the rumen microbial composition can be traced back to the first weeks of life. The undeveloped rumen is initially inoculated with aerobic and facultative anaerobic bacteria, which are gradually replaced by exclusive anaerobic bacteria [134], methanogenic archaea, anaerobe fungi and ciliate protozoa [135]. The rumen microbial community is established by random colonization from the surrounding metacommunity, including the vagina, skin during suckling and grooming, bacteria in colostrum, milk and later in the feed and drinking water and aerosolized bacteria [405,406]. The ambient conditions in the rumen will be beneficial for the proliferation of some bacteria whereas others will wash out. The dynamic steady-state of the mature rumen microbiome has a core community that is therefore shaped by host-specific influences and early life events such as rearing and weaning strategies, medical interventions and diet types.

In our study, the host-specificity of the rumen microbial communities was investigated by a rumen content transfer. Completely emptying the rumen and replacing its content with that

of another animal, with another host-specific composition, provokes a profound disturbance of the rumen ecosystem. This drastic disturbance allowed to examine the extent to which host-specific factors influence and reshape the microbial community while it strives for a new dynamic stability (Chapter 3). Following this transfer, two cows suffered from decreased feed intake which affected the rumen ecosystem and consequently reduced the milk and methane production. This "stress" response caused the data of these cows to be not comparable to the other two cows, which complicated the statistical analysis. Fortunately, the samples collected during this stress response provided an unique insight in the resilience of the rumen microbiome and the way in which the microbial community is restored. The two cows that did not suffer a temporary feeding depression, reacted differently on the rumen content transfer. During the two days following the transfer, the rumen microbial ecosystem evolved from the adopted microbial community profile of the donor cow (which was retained during the first two days after transfer) to a new dynamic steady-state. In fact, despite the very divergent course of events, both the two cows that suffered a stress response and the two cows that did not, achieved the same: a new dynamic, steady-state microbial community, that neither resembled the microbial profile of the donor nor was the before-transfer profile of the host restored. These results indicate that the rumen ecosystem relies on a vast biosphere of transient and low abundant species to maintain and/or restore the microbial ecosystem after severe perturbations. Following such a perturbation (like the rumen content transfer), the final community composition is unpredictable, and thus not mainly determined by host-related influences.

While the individual host effect was not clearly observed during the rumen content transfer experiment, the rumen microbial and fermentative differences between two distinct breeds were explicitly observed when comparing heifers of the Holstein-Friesian breed with heifers from the Double-muscled Belgian Blue breed. Both breeds showed distinct rumen community compositions as indicated by beta-diversity analysis, suggesting that breed-specific factors (such as physiological characteristics and early-life events) can uniformly influence and shape the bacterial community composition of the rumen. Beside the breed-effect; this experiment demonstrated another factor that seemed to significantly influence the rumen microbial community: sample time. The community profiles demonstrated a sample time-specific composition, emphasizing that single sample collection per cow (in this case with an oral probe) only provides a snapshot of the community. Presumably these results may indicate that the microbial community composition is also subject to diurnal fluctuations, similar to the rumen pH, VFA concentrations and methane production levels.

Different and somewhat contrasting views exist on the assembly of microbial communities. Curtis and Sloan (2004) argued that the local bacterial community structure is regulated by the size and diversity of surrounding metacommunities. Accordingly, the local community composition is a product of random events in connection to the recruitment of functionally equivalent bacteria from the source community [405,407]. Applied to the rumen environment, the active rumen microbiome is regulated by the constant influx of bacteria and Archaea with aerosols, feed, drinking water and saliva, and the constant efflux of bacteria from the liquid phase. Because of the constant influx of bacteria, as well as the random nature of bacterial colonization and the dominance of heterotrophic "generalist" bacteria, the community structure is hypothesized to be independent from the environment [407]. Opposite to this view stands another popular hypothesis, based on the famous ecological tenet of Baas-Becking and Beijernick: "Everything is everywhere, but the environment selects" [408]. The idea is that microorganisms are ubiquitously distributed and the local microbial community is selected from this vast pool of species by the prevailing environmental conditions. The rumen transfer experiment suggests that the rumen microbial community is divergent and not observably influenced by host-related factors, reminiscent of the view of Curtis and Sloan (2004). On the other hand, the comparison of HF versus DMBB suggests that the breed-specific characteristics can actively select bacteria that can thrive in the rumen environment, following the hypothesis of Baas-Becking and Beijernick. Presumably, the truth lies in the middle: The prevailing conditions of the rumen, influenced by host and breed specific factors, diet type, living conditions, etc. will exert a selection on the microbes in the rumen and determine the microbial community structure. However, specofoc selection criteria might retain different generalist species equally able to cope with the environmental conditions or the nutrition source.

The rumen microbial community consists of a core bacterial community [76,103], complemented with a vast biosphere of low abundant (often specialists) and transient species and operates at several trophic levels. The divers rumen community harbors extensive redundancy due to the presence of numerous coexisting species performing similar functions, providing resilience against community disturbances. The redundancy is best illustrated by generalists such as *Prevotella* and *Butyrivibrio*, able to thrive in a wide range of environmental conditions and food sources. As a consequence, the levels of fundamental fermentation metrics ($VFA/NH_3^+/CH_4$ concentrations, pH) may be unrelated to the microbial community composition, which is further supported by several studies that observed considerable compositional changes in the rumen microbial communities, while rumen fermentation metrics remained unaltered [409,410]. Nevertheless, abrupt or fundamental changes can forcedly alter

both the rumen microbial community structure as well as its activity. The transition to a new microbial steady-state might take several days or even weeks [332,411] whereas the microbial activity may shift more rapidly. Shifts in activity are carried by those species capable of using a variety of resources, i.e. generalists. Furthermore, recent studies have shown that presence and abundance does not necessarily imply activity [73,412,413]. DNA-based sequencing (16S rRNA metabarcoding) helps to observe the total community, but is limited as it cannot distinguish if genes stem from active cells, inactive cells or dead/lysed cells. Due to the presumed lag period between functional changes and taxonomic changes, and the inability of DNA-based metabarcoding to identify active members, meta-transcriptomics may provide a better understanding of the microbial community composition in relation to its activity.

In sharp contrast to the bacterial population, the methanogens are characterized by a very low richness. In the studies described in this PhD dissertation, the observed methanogen community consisted usually between 15 and 20 OTUs. Presumably, this phylogenetic "diversity" is owed to environment adaptation [98], which is further supported by the metabarcoding of samples collected from the three rumen environments (Chapter 2), where distinct methanogen communities were observed in the different environments. The absolute winners of the communities were *Methanobrevibacter gottschalkii*, *Mbb. ruminantium* and *Methanosphaera*, who dominated the rumen methanogen communities in all environments. Despite this low diversity, the methanogens maintain a stable community with high resilience, as was concluded from the various experiments. No distinct differences were observed between the methanogen community in the rumen of HF and DMBB heifers nor between sampling moment. The two cows that suffered from feed intake depression after the rumen content transfer, had no altered methanogen community compositions. Presumably, the methanogens owe their stability to the specificity and stability of the niche they occupy. According to Janssen (2008), methanogenesis is directly resulting from the formation of H_2 [98]. The continuous formation of H_2 in the rumen ecosystem assures the methanogens of a stable influx of reducing equivalents for which they have no competition.

7.2.2 Conclusion and perspectives

The microbial community structure is influenced by host and breed specific factors, but mainly determined by diet composition and physiological stage. In spite of these determinants of the community composition and activity, rumen microbiota are comparable between individual cows due to the common core of omnipresent species and genera that dominate the rumen

ecosystems [103]. A large part of the research on ruminant animals aims for lower methane production levels, without reducing production performances. However, our results have indicated that the methanogen community is resilient and imperturbable in terms of taxonomic composition and absolute abundances, as it remained unaffected by breed and host effects, varying nutritional conditions and even severe perturbations of the microbial ecosystem. Opposite to the community structure, the activity (i.e. methanogenesis) proved to be more dynamic and heavily influenced by various factors (cfr. differences in methane yield under variable DMI). These findings suggest that acting directly on the methanogen activity, rather than on the methanogen community composition or abundance, has the most potential to mitigate enteric methane emissions.

Methods such as early life programming (using feed additives and pre/probiotics), breeding programs or genetic selection can influence how the rumen microbial community is formed and can improve feed efficiency. However, these strategies are subject to host and breed variability and will only be moderately successful in mature animals. Immunization against rumen methanogens by vaccination posed a promising strategy for methane mitigation as it requires only a one-time vaccination. However for the moment, the obtained reduction of methane emissions is limited (or even completely absent) as the vaccination targets specific species, while methanogenesis is continued by other methanogens [414–416].

Given that enteric methane emissions can be significantly reduced by up to 60% without compromising DMI, milk yield or growth [417,418], we find that inhibiting growth and activity of methanogens provides the best course of action to approximate the theoretical maximum of methane reductions. Therefore, promising mitigation strategies involve inhibitory compounds with proven *in vivo* effectiveness (ex. 3NOP, essential oils, PUFAs, etc.). Unfortunately, continuation of treatment is usually necessary to maintain minimum methane emissions and treatments are often costly. In the near future, the focus must be on both identifying novel (and cheap) mitigating strategies aimed at inhibiting methanogenesis (independent of its taxonomic origin) and investigating the long-term efficacy and the on-farm usability.

7.3 STUDYING THE EFFECT OF CROSS-CONTAMINATION AND ITS EFFECT ON THE PIG'S INTESTINAL MICROBIAL ECOSYSTEM

7.3.1 The persistence and widespread occurrence of tetracyclines

Tetracyclines are a class of popular pharmaceutical compounds derived from tetracycline, a secondary metabolite of some *Streptomyces* species. Already within a year after the discovery of tetracycline, the first evidence of bacterial resistance against the drug was reported [419]. The frequent use of tetracyclines in human and veterinary medicine has contributed greatly to the widespread emergence of ribosome and efflux-based resistance. Our results clearly indicated high levels of tetracycline resistance in the microbial communities of the pig's intestinal ecosystems, even in the absence of antimicrobial compounds. This is likely due to the frequent use of tetracyclines in agriculture, as well as the persistence of tetracycline resistance in microbial ecosystems and the natural incidence of tetracycline resistance genes in microbial communities.

Resistome analysis was performed to identify common *tet* genes in the intestinal environment. A reactor sample was collected during doxycycline treatment, circular plasmid DNA was isolated and used for shotgun sequencing. Subsequent data processing identified *tet*(Q), *tet*(O) and *tet*(W) as most common *tet* genes in the intestinal environment (data not shown). Based on these results, specific *tet* genes were selected for subsequent quantification with qPCR. During the *in vivo* trials, the control pigs (who did not receive any treatment) had an average of 0.4% *tet*(Q) and 1.8% *tet*(O) relative to 16S gene copies. Even in the cecal ecosystem of organically raised pigs, which were used to inoculate the *in vitro* cecal microbial ecosystem, the relative abundances of these genes was 1.4% and 1.3% relative to 16S gene copies. During the start-up phase of the reactors simulating the cecal microbial ecosystem, the microbial community maintained similar relative abundances (1.1% and 0.7% respectively) of *tet*(Q) and *tet*(O). The stable persistence of these high levels of *tet* genes is remarkable, not only because of the complete absence of any selection pressure imposed by an antimicrobial compound but also because the reactor conditions select for fast growing bacteria. This confirms that the acquisition of mobile resistance genes does not always impose a metabolic burden at the expense of the growth rate [225,420].

7.3.2 The influences of sub therapeutic doxycycline on the pigs intestinal microbial community

The *in vitro* reactor setup was proven to be a worthy alternative for *in vivo* experiments and was used to investigate the effects of 1, 4 or 16 mg l^{-1} doxycycline hyclate on the pig's cecal microbial community. During administration of these concentrations, the bacterial richness and diversity did not decrease and also the beta-diversity remained unaltered. On the one hand, 1, 4 or 16 mg l^{-1} doxycycline hyclate could have induced a community disturbance by inhibiting the growth of sensitive bacteria. Mainly dominant taxa could have been affected due to their higher numerical proportions in the community. The niches they occupy would become (partially) available for other species and transient bacteria would get the opportunity to gain dominance and occupy these niches. Furthermore bacteria in biofilms or attached to fibers are less affected by the inhibitory impact of doxycycline. We hypothesize that coincidence determines which bacteria are affected by doxycycline and which are given the opportunity to proliferate. Because of this random nature, the possible response could not have been observed in ordination plots or heatmaps as it would be hidden behind the dynamic fluctuations naturally occurring in the community and the specificity of the community in each reactor run. On the other hand, it is also possible that administration of doxycycline did not influence the taxonomic compositions. This would imply that resistant species replaced the sensitive species within the same taxa, thus maintaining their relative abundances. If this hypothesis is correct, it would mean that resistance is widespread and abundantly represented in the entire community.

It is a possibility that metabarcoding fails at identifying possible taxonomic changes induced by doxycycline administration (both *in vivo* and *in vitro*) due to the intrinsic nature of PCR-based metagenomics. The increased relative abundance of one species will indivertibly result in a decreased relative abundance of others, although their absolute abundance might not have changed. Metabarcoding alone might not be the best tactic to examine the influence of an antibiotic treatment on a microbial community. Abundances generated by this technique are semi-quantitative at best and the observed dynamics might not reflect those of the actual taxon densities [421,422]. For example, Daniels et al. (2013) found that antibiotic treatment to target pathogen *Pseudomonas aeruginosa* in cystic fibrosis patients, resulted in an increased relative abundance of *P. aeruginosa* and a decreased relative abundance of non-pseudomonal species. QPCR revealed that antibiotic treatment did not induce an actual increase of pathogens, instead both *P. aeruginosa* and non-pseudomonal species decreased but the relative decrease of non-pseudomonal species was larger [423]. The authors only studied two groups, making the results

easier to put into context. However, we investigated more complex microbial communities, represented by hundreds of species with complex inter-bacterial interactions and dependencies, making it difficult, not to say impossible, to link shifts in relative abundance to changes in abundances of individual taxa. Metabarcoding is therefore mainly informative on microbial compositional changes (mainly absence or presence of taxa), but only limited to measuring changes in the abundance of these taxa (over time) because the relative abundances reported by metabarcoding is not (always) related to the absolute abundances of the taxa in an environment. Therefore, making conclusions based on the relative abundance data could lead to erroneous interpretations. QPCR assays to quantify specific taxonomic groups, in parallel with metabarcoding, can help examine changes in the taxonomic composition over time or in response to antibiotics [422]. As qPCR is only moderately sensitive (can only separate twofold changes in gene concentration) and suffers from specific limitations such as data representation (copies per ml sample, per ng DNA extract or relative to a reference gene), primer specificity and PCR efficiency, Props et al. (2016) proposed single-cell enumeration with flow cytometry to determine the absolute taxon abundances from the compositional data obtained with metabarcoding [421]. Alternatively, Stämmler (2016) described a method to obtain quantities measured from metabarcoding without relying on external technologies. By spiking controlled amounts of exogenous bacteria into crude samples, the read counts of endogenous bacteria can be rescaled after sequencing [424].

Despite the lack of changes in the taxonomic profiles in response to doxycycline administration, selective plate cultivation did provide strong and consistent evidence of the influence of doxycycline on the enrichment of some resistant cultivable taxa. These results are in contrast to the limited influence of even the highest concentration of doxycycline on the absolute abundances of specific *tet* genes. This could be a consequence of the limitations of culture-resistant cultivable bacteria, which views only a small part of the community and does not give information on taxonomy and the type of resistance. Especially prominent community members (species from the Bacteroidetes and Proteobacteria) were missed, meaning that their response to DOX administration is unknown. qPCR analysis provides information about the absolute abundance of specific resistance gene groups, but not about their taxonomic distribution. In addition, only the abundances of a few specific *tet*-genes were quantified, while other resistance genes might additionally play an important role in the ecosystem, despite their low individual occurrences. Although traditional agar plate cultivation and qPCR assays are an obvious choice in these types of experiments, they provide limited information and interpretation should be careful. Developments in the field of metagenomics can address these

shortcomings. Hi-C sequencing, based on DNA crosslinking in living cells prior to NGS library preparation, is able to reliably associate plasmids with each other and with the chromosomal DNA of the host cell [385]. A recently described technique called epicPCR isolates bacterial cells in emulsion beads and uses fusion PCR to physically link specific functional and phylogenetic genes prior to amplicon sequencing [386]. In the near future, these novel techniques could be used to correlate taxonomic classification and resistance mechanisms of the species in the community.

7.3.3 The hidden dangers of cross-contamination

Antibiotic use in intensive agriculture is a contributor to the clinical problems of antimicrobial resistance in human medicine. In the Belgian pig husbandry, antibiotics are mainly mixed into the feed mix at the feed mill and administered as feed additive. The production process, transport and storage on the farm can lead to cross-contamination of antimicrobial compounds from medicated feeds to non-medicated feeds. As a result of this carry-over, the intestinal bacteria can become unintentionally exposed to low concentrations of antibiotics. Screening experiments where feeds and fecal samples are collected (preferably at farm level or the abattoir) and screened for the presence of antibiotic compounds can be used to investigate the current cross-contamination prevalence and evaluate the effectiveness of the measures to restrict carry-over. In the past, such studies have been performed [156,157,296] but many of these findings and conclusions are no longer relevant due to new legislations to restrict cross-contamination. In the *CrossContam* project, financed by the Federal Public Service (FOD) for health, food chain safety and environment, experiments were conducted to estimate the prevalence of cross-contamination [159], to determine the intestinal and fecal concentrations of antibiotic residues in pigs fed cross-contaminated feed [173], to investigate the potential of these residues to promote transfer frequency of resistance plasmids [427] and influence the microbial community and increase abundances of resistance genes and resistant species (Chapter 5 and 6).

We investigated the effects of the intestinal concentrations of doxycycline on the *in vitro* simulated microbial ecosystem of the pig's cecum. The administration of even the lowest concentrations doxycycline had the potential to enrich resistant bacteria. This was best observed with the selective cultivation of total and doxycycline-resistant *Escherichia coli*. The administration of 1 mg l^{-1} doxycycline hyclate (corresponding to a concentration of 0.87 mg l^{-1} doxycycline, which is the active component), resulted in a fivefold increase of resistant *E. coli* and a thousand fold increase was observed after administration of 4 (3.47) mg l^{-1} doxycycline

hyclate. These results clearly indicate that carry-over of doxycycline in pig feed can enrich resistant bacteria in the intestinal tract of pigs, even in spite of the already high levels of tetracycline resistance in the intestinal ecosystem. These resistant bacteria (and the antibiotic residues) are excreted in the feces, which is often used as organic fertilizer for agricultural crops. As such, cross-contamination contributes to the spread of antibiotic resistance in the environment.

Obviously, this conclusion is only based on our *in vitro* experiments, which only investigated the influence of doxycycline. Though doxycycline is a frequently administered antibiotic in pig husbandry, many other antibiotics are used in livestock cultivation (such as chlor -and oxytetracycline, sulfadiazine, tylosin and amoxicillin) and further research to the influence of carry-over levels of these antibiotics is necessary to understand the complete risk of cross-contamination.

7.3.4 Conclusion and perspectives

Many legislative restrictions have been imposed to reduce the (unnecessary) administration of antibiotics or to limit cross-contamination. But despite these efforts, frequent antibiotic use remains omnipresent in livestock production and concomitantly maintaining cross-contamination to non-target feed. These antibiotic compounds can influence the microbial community and selects resistant bacteria in the intestinal microbial ecosystem. Our *in vitro* experiments indicated that doxycycline residues, as a result of 3% carry-over from a medicated feed, can influence the microbial activity and enrich specific tetracycline-resistant species. It becomes clear that an antibiotic-free animal husbandry is utopian. Instead of only focusing on diminishing the massive antibiotic use for livestock, perhaps an effort should also be made to restrict the spread of antibiotic residues and resistant bacteria to the environment by implementing a smarter animal waste management and alternative disease prevention methods. Antibiotic use can be reduced on closed pig farms by (partly) substituting antimicrobial treatments with vaccination programs and biosafety measures, without hurting the profit margins [428,429]. Good practices for biosafety can be the first line of defense against disease outbreaks by implementing measures to achieve three goals: segregation (surveillance of the herd and quarantining potentially infected animals away from uninfected animals and material or euthanizing infected pigs), sanitation (procedures for regular cleaning and disinfection of housing and transportation facilities, materials) and external biosecurity (controlling entry and exit points of people, animals and supplies on the pig production facilities). These measures form the cornerstone of herd health maintenance, but are more effective in conjunction with

vaccination. Vaccines are commercially available for a wide range of bacterial and viral infections (porcine parvovirus, colibacillosis, leptospirosis, erysipelas, mycoplasma pneumonia, actinobacillosis, etc.). Furthermore, custom-made vaccines from a pathogen isolated from diseased pigs, so called autogenous vaccines, provide high specificity of vaccine treatments. Farmers could be convinced to invest in vaccination programs and implementation of biosecurity by promoting its benefits, spreading knowledge (study days, on-farm consultancy) and governmental subsidies.

Programs and initiatives (such as vaccination) exist to lower antibiotic use on the farms though preventive and curative treatment may still be required under certain circumstances. Currently, the manure of antibiotic-treated animals is collected in the same basement with the manure of healthy animals, and is later used as fertilizer. It could be feasible to design new stables with multiple manure basements in order to separate manure from treated and non-treated pigs and thus also separate the downstream waste processing to avoid the spread of antibiotic residues and resistant bacteria to the environment.

7.4 GENERAL CONCLUSION

At the moment, intensive livestock production strives for maximum production with a minimum of costs, and although the effects are not always directly observable, agricultural practices contribute profoundly to the current issues of climate change and the increasing prevalence of antibiotic resistance. Enteric methane production by cattle plays a role in greenhouse gas emissions and the frequent prophylactic and metaphylactic administration of antibiotics to livestock animals selects for resistant bacteria in the intestinal tract and in the feces.

These problems are inherent to livestock cultivation because farmers have no incentive to change their established customs, but ultimately, it is the consumer's demands that determines the quality and the price of the product. The mindset and opinion in regard to meat (and milk) consumption is changing (mostly in western countries). Massive consumption of animal-derived products is shifting towards vegetarian and/or vegan alternatives due to raised awareness about animal welfare and ecological impact. Investing in raising awareness of the ecological impact and long-term consequences amongst consumers, farmers and governments could on the one hand lower the demand for animal-derived products and thereby lower the pressure for massive livestock production, and on the other hand coerce livestock farmers to implement more eco-friendly production systems. For example, invest in higher hygienic and

preventive measures or vaccinations to replace antibiotic treatments and administer methanogenesis-inhibiting compounds (as dietary additive) to reduce methane emission levels.

References

REFERENCES

[1] FAOSTAT, Food and Agriculture Data, (n.d.). http://www.fao.org/faostat/en/#home (accessed July 19, 2017).

[2] MIRA, Milieurapport Vlaanderen: Emissie van broeikasgassen door de landbouw, (2016). http://www.milieurapport.be/nl/feitencijfers/sectoren/landbouw/emissies-naar-lucht-door-de-landbouw/emissie-van-broeikasgassen-door-de-landbouw/ (accessed July 19, 2017).

[3] D. Klemm, B. Heublein, H.-P. Fink, A. Bohn, Cellulose: Fascinating Biopolymer and Sustainable Raw Material, Angew. Chemie Int. Ed. 44 (2005) 3358–3393. doi:10.1002/anie.200460587.

[4] C.B. Rodrigue, N.N. Aixen, The effect of fine grinding of hay on the ration digestibility, rate of passge, and fat content of milk, Can. J. Anim. Sci. 40 (1960) 23–29. doi:10.4141/cjas60-004.

[5] G.F. Hartnell, L.D. Satter, Determination of rumen fill, retention time and ruminal turnover rates of ingesta at different stages of lactation in dairy cows., J. Anim. Sci. 48 (1979) 381–92.

[6] J.B. Russell, Rumen Microbiology and its Role in Ruminant Nutrition, Ithaca, N.Y., 2002.

[7] W.Z. Yang, K.A. Beauchemin, Effects of Physically Effective Fiber on Chewing Activity and Ruminal pH of Dairy Cows Fed Diets Based on Barley Silage, J. Dairy Sci. 89 (2006) 217–228. doi:10.3168/jds.S0022-0302(06)72086-0.

[8] R.I. Mackie, C.S. McSweeney, R.I. Aminov, Rumen, in: Encycl. Life Sci., John Wiley & Sons, Ltd, Chichester, UK, 2001. doi:10.1038/npg.els.0000404.

[9] T.G. Nagaraja, Microbiology of the Rumen, in: Rumenology, Springer International Publishing, Cham, 2016: pp. 39–61. doi:10.1007/978-3-319-30533-2_2.

[10] J.C. McCann, T.A. Wickersham, J.J. Loor, High-throughput Methods Redefine the Rumen Microbiome and Its Relationship with Nutrition and Metabolism., Bioinform. Biol. Insights. 8 (2014) 109–25. doi:10.4137/BBI.S15389.

[11] P.J. Weimer, Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations, Front. Microbiol. 6 (2015) 296. doi:10.3389/fmicb.2015.00296.

[12] M.J. Latham, M.J. Wolin, Fermentation of cellulose by Ruminococcus flavefaciens in the presence and absence of Methanobacterium ruminantium, Appl. Environ. Microbiol. 34 (1977) 297–301.

[13] S.G. Pavlostathis, T.L. Miller, M.J. Wolin, Kinetics of Insoluble Cellulose Fermentation by Continuous Cultures of Ruminococcus albus., Appl. Environ. Microbiol. 54 (1988) 2660–2663. doi:10.1007/BF00170581.

[14] M.P. Bryant, N. Small, The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen., J. Bacteriol. 72 (1956) 16–21.

[15] G. Suen, P.J. Weimer, D.M. Stevenson, F.O. Aylward, J. Boyum, J. Deneke, C. Drinkwater, N.N. Ivanova, N. Mikhailova, O. Chertkov, L.A. Goodwin, C.R. Currie, D. Mead, P.J. Brumm, The complete genome sequence of fibrobacter succinogenes s85 reveals a cellulolytic and metabolic specialist, PLoS One. 6 (2011). doi:10.1371/journal.pone.0018814.

[16] F. Chaucheyras-Durand, S. Masséglia, G. Fonty, E. Forano, Influence of the Composition of the Cellulolytic Flora on the Development of Hydrogenotrophic Microorganisms, Hydrogen Utilization, and Methane Production in the Rumens of Gnotobiotically Reared Lambs, Appl. Environ. Microbiol. 76 (2010) 7931–7937. doi:10.1128/AEM.01784-10.

[17] A. Gasparic, J. Martin, A.S. Daniel, H.J. Flint, A xylan hydrolase gene cluster in Prevotella ruminicola B(1)4: sequence relationships, synergistic interactions, and oxygen sensitivity of a novel enzyme with exoxylanase and beta-(1,4)-xylosidase activities., Appl. Environ. Microbiol. 61 (1995) 2958–64. http://www.ncbi.nlm.nih.gov/pubmed/7487028 (accessed March 3, 2017).

[18] K. Miyazaki, J.. Martin, R. Marinsek-Logar, H.. Flint, Degradation and Utilization of Xylans by the Rumen AnaerobePrevotella bryantii(formerlyP. ruminicolasubsp.brevis) B14, Anaerobe. 3 (1997) 373–381. doi:10.1006/anae.1997.0125.

[19] A.G. Williams, S.E. Withers, The regulation of xylanolytic enzyme formation by Butyrivibrio fibrisolvens NCFB 2249, Lett. Appl. Microbiol. 14 (1992) 194–198. doi:10.1111/j.1472-765X.1992.tb00683.x.

[20] W.J. Kelly, S.C. Leahy, E. Altermann, C.J. Yeoman, J.C. Dunne, Z. Kong, D.M. Pacheco, D. Li, S.J. Noel, C.D. Moon, A.L. Cookson, G.T. Attwood, The Glycobiome of the Rumen Bacterium Butyrivibrio proteoclasticus B316T Highlights Adaptation to a Polysaccharide-Rich Environment, PLoS One. 5 (2010) e11942. doi:10.1371/journal.pone.0011942.

[21] H.J. Strobel, Vitamin B12-dependent propionate production by the ruminal bacterium Prevotella ruminicola 23., Appl. Environ. Microbiol. 58 (1992) 2331–3. http://www.ncbi.nlm.nih.gov/pubmed/1637169 (accessed March 3, 2017).

[22] J. Lou, K.A. Dawson, H.J. Strobel, Glycogen biosynthesis via UDP-glucose in the ruminal bacterium Prevotella bryantii B1(4)., Appl. Environ. Microbiol. 63 (1997) 4355–9. http://www.ncbi.nlm.nih.gov/pubmed/9361422 (accessed March 2, 2017).

[23] N.O. Van Gylswyk, Some Aspects of the Metabolism of Butyrivibrio fibrisolvens, J. Gen. Microbiol. 97 (1976) 105–111. doi:10.1099/00221287-97-1-105.

[24] G. Attwood, K. Reilly, B. Patel, Clostridium proteoclasticum sp. nov., a Novel Proteolytic Bacterium from the Bovine Rumen, Int. J. Syst. Bacteriol. 46 (1996) 753–758.

[25] M. Marounek, D. Duskova, Metabolism of pectin in rumen bacteria Butyrivibrio fibrisolvens and Prevotella ruminicola, Lett. Appl. Microbiol. 29 (1999) 429–433. doi:10.1046/j.1472-765X.1999.00671.x.

[26] D. Dusková, M. Marounek, Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rumen bacterium Lachnospira multiparus., Lett. Appl. Microbiol. 33 (2001) 159–63. http://www.ncbi.nlm.nih.gov/pubmed/11472526 (accessed March 3, 2017).

[27] R.E. Hungate, The Rumen and Its Microbes., Academic Press, New York, 1966.

[28] M. Wojciechowicz, A. Ziołecki, A note on the pectinolytic enzyme of Streptococcus bovis., J. Appl. Bacteriol. 56 (1984) 515–8. http://www.ncbi.nlm.nih.gov/pubmed/6746470 (accessed March 3, 2017).

[29] S.M. O'herrin, W.R. Kenealyl, Glucose and Carbon Dioxide Metabolism by Succinivibrio dextrinosolvens, Appl. Environ. Microbiol. 59 (1993) 748–755.

[30] J.R. Russell, T. Hino, Regulation of lactate production in Streptococcus bovis: A spiraling effect that contributes to rumen acidosis., J. Dairy Sci. 68 (1985) 1712–21..

[31] G. Avgustin, R.J. Wallace, H.J. Flint, Phenotypic diversity among ruminal isolates of Prevotella

ruminicola: proposal of Prevotella brevis sp. nov., Prevotella bryantii sp. nov., and Prevotella albensis sp. nov. and redefinition of Prevotella ruminicola., Int. J. Syst. Bacteriol. 47 (1997) 284–8. doi:10.1099/00207713-47-2-284.

[32] M.A. Cotta, Amylolytic Activity of Selected Species of Ruminal Bacteria, Appl. Environ. Microbiol. 54 (1988) 772–776. https://naldc.nal.usda.gov/download/27607/PDF (accessed March 3, 2017).

[33] A. Castillo-González, M. Burrola-Barraza, J. Domínguez-Viveros, A. Chávez-Martínez, Rumen microorganisms and fermentation, Arch. Med. Vet. 46 (2014) 349–361. doi:10.4067/S0301-732X2014000300003.

[34] E. Stackebrandt, R. Hespell, The Family Succinivibrionaceae, in: Prokaryotes Vol. 3 Archaea. Bact. Firmicutes, Actinomycetes, 2006.

[35] M.P. Bryant, N. Small, C. Bouma, H. Chu, Bacteroides ruminicola n. sp. and Succinimonas amylolytica; the new genus and species; species of succinic acid-producing anaerobic bacteria of the bovine rumen., J. Bacteriol. 76 (1958) 15–23.

[36] H.-T. Wang, J.-T. Hsu, Optimal protease production condition for Prevotella ruminicola 23 and characterization of its extracellular crude protease, Anaerobe. 11 (2005) 155–162. doi:10.1016/j.anaerobe.2004.10.007.

[37] T. Blackburn, Protease Production by Bacteroides amylophilus Strain H 18, J. Gen. Microbiol. 53 (1968) 27–36.

[38] M.A. Cotta, R.B. Hespell, Proteolytic activity of the ruminal bacterium Butyrivibrio fibrisolvens., Appl. Environ. Microbiol. 52 (1986) 51–8.

[39] J.B. Russell, Fermentation of Peptides by Bacteroides ruminicola B(1)4., Appl. Environ. Microbiol. 45 (1983) 1566–74.

[40] N.R. Krieg, J.T. Staley, D.R. Brown, B.P. Hedlund, B.J. Paster, N.L. Ward, W. Ludwig, W.B. Whitman, eds., Bergey's Manual of Systematic Bacteriology: Volume 4: The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentispaerae, Verrucomicrobia, Chlamydiae and Planctomycetes, second edi, 2011.

[41] J.B. Russell, Fermentation of Cellodextrins by Cellulolytic and Noncellulolytic Rumen Bacteria, Appl. Environ. Microbiol. 49 (1985) 572–576..

[42] T.B. Stanton, E. Canale-Parola, Treponema bryantii sp. nov., a rumen spirochete that interacts with cellulolytic bacteria., Arch. Microbiol. 127 (1980) 145–56.

[43] R. Prabhu, E. Altman, M.A. Eiteman, Lactate and acrylate metabolism by Megasphaera elsdenii under batch and steady-state conditions., Appl. Environ. Microbiol. 78 (2012) 8564–70. doi:10.1128/AEM.02443-12.

[44] S.H.G. Allen, Lactate-oxaloacetate transhydrogenase from Veillonella alcalescens, 89 (1982) 367–376. doi:10.1016/S0076-6879(82)89066-6.

[45] C.C. Scheifinger, M.J. Wolin, Propionate formation from cellulose and soluble sugars by combined cultures of Bacteroides succinogenes and Selenomonas ruminantium., Appl. Microbiol. 26 (1973) 789–95.

[46] K. Denger, B. Schink, Energy conservation by succinate decarboxylation in Veillonella parvula, J. Gen. Microbiol. 138 (1992) 967–971. doi:10.1099/00221287-138-5-967.

[47] N.O. van Gylswyk, Succiniclasticum ruminis gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism., Int. J. Syst. Bacteriol. 45 (1995) 297–300. doi:10.1099/00207713-45-2-297.

[48] E. Jami, I. Mizrahi, F. Bushman, T. DeSantis, G. Andersen, Composition and Similarity of Bovine Rumen Microbiota across Individual Animals, PLoS One. 7 (2012) e33306. doi:10.1371/journal.pone.0033306.

[49] D.M. Stevenson, P.J. Weimer, Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR, Appl. Microbiol. Biotechnol. 75 (2007) 165–174. doi:10.1007/s00253-006-0802-y.

[50] D.W. Pitta, W.E. Pinchak, S.E. Dowd, J. Osterstock, V. Gontcharova, E. Youn, K. Dorton, I. Yoon, B.R. Min, J.D. Fulford, T.A. Wickersham, D.P. Malinowski, Rumen Bacterial Diversity Dynamics Associated with Changing from Bermudagrass Hay to Grazed Winter Wheat Diets, Microb. Ecol. 59 (2010) 511–522. doi:10.1007/s00248-009-9609-6.

[51] A. Ramsak, M. Peterka, K. Tajima, J.C. Martin, J. Wood, M.E.A. Johnston, R.I. Aminov, G. Avgustin, Unravelling the genetic diversity of ruminal bacteria belonging to the CFB phylum., FEMS Microbiol. Ecol. 33 (2000) 69–79.

[52] H. Matsui, K. Ogata, K. Tajima, M. Nakamura, T. Nagamine, R.I. Aminov, Y. Benno, Phenotypic Characterization of Polysaccharidases Produced by Four Prevotella Type Strains, Curr. Microbiol. 41 (2000) 45–49. doi:10.1007/s002840010089.

[53] J. Miron, D. Ben-Ghedalia, M. Morrison, Invited review: adhesion mechanisms of rumen cellulolytic bacteria., J. Dairy Sci. 84 (2001) 1294–309. doi:10.3168/jds.S0022-0302(01)70159-2.

[54] A.E. Naas, A.K. Mackenzie, J. Mravec, J. Schückel, W.G.T. Willats, V.G.H. Eijsink, P.B. Pope, Do rumen Bacteroidetes utilize an alternative mechanism for cellulose degradation?, MBio. 5 (2014) e01401-14. doi:10.1128/mBio.01401-14.

[55] E. Stackebrandt, H. Hippe, Transfer of Bacteroides amylophilus to a new genus Ruminobacter gen. nov., nom. rev. as Ruminobacter amylophilus comb. nov., Syst. Appl. Microbiol. 8 (1986) 204–207. doi:10.1016/S0723-2020(86)80078-9.

[56] R.R. Gokarn, M.A. Eiteman, S.A. Martin, K.-E.L. Eriksson, Production of succinate from glucose, cellobiose, and various cellulosic materials by the ruminai anaerobic bacteria Fibrobacter succinogenes and Ruminococcus flavefaciens, Appl. Biochem. Biotechnol. 68 (1997) 69–80. doi:10.1007/BF02785981.

[57] B.J. Paster, E. Canale-Parola, Physiological diversity of rumen spirochetes., Appl. Environ. Microbiol. 43 (1982) 686–93. http://www.ncbi.nlm.nih.gov/pubmed/7073277 (accessed March 10, 2017).

[58] T.H. Blackburn, R.E. Hungate, Succinic acid turnover and propionate production in the bovine rumen., Appl. Microbiol. 11 (1963) 132–5.

[59] S. Sawanon, Y. Kobayashi, Synergistic fibrolysis in the rumen by cellulolytic Ruminococcus flavefaciens and non-cellulolytic Selenomonas ruminantium: Evidence in defined cultures, Anim. Sci. J. 77 (2006) 208–214. doi:10.1111/j.1740-0929.2006.00339.x.

[60] H. Kudo, K.J. Cheng, J.W. Costerton, Interactions between Treponema bryantii and cellulolytic bacteria in the in vitro degradation of straw cellulose., Can. J. Microbiol. 33 (1987) 244–8.

[61] B.A. Dehority, H.W. Scott, B.A. Dehority, L.V. Packett, Cecelia, I.M. Robinson, Extent of Cellulose and Hemicellulose Digestion in Various Forages by Pure Cultures of Rumen Bacteria,

J. Dairy Sci. 50 (1967) 1136–1141. doi:10.3168/jds.S0022-0302(67)87579-9.

[62] M. Fondevila, B.A. Dehority, Interactions between Fibrobacter succinogenes, Prevotella ruminicola, and Ruminococcus flavefaciens in the digestion of cellulose from forages., J. Anim. Sci. 74 (1996) 678–684. doi:/1996.743678x.

[63] N. ASANUMA, T. HINO, Regulation of fermentation in a ruminal bacterium, Streptococcus bovis, with special reference to rumen acidosis, Anim. Sci. J. 73 (2002) 313–325. doi:10.1046/j.1344-3941.2002.00044.x.

[64] P.C. Aikman, P.H. Henning, D.J. Humphries, C.H. Horn, Rumen pH and fermentation characteristics in dairy cows supplemented with Megasphaera elsdenii NCIMB 41125 in early lactation, J. Dairy Sci. 94 (2011) 2840–2849. doi:10.3168/jds.2010-3783.

[65] M. Marounek, S. Bartos, Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch., J. Appl. Bacteriol. 63 (1987) 233–8. http://www.ncbi.nlm.nih.gov/pubmed/3429358 (accessed March 12, 2017).

[66] J.D. Evans, S.A. Martin, Factors Affecting Lactate and Malate Utilization by Selenomonas ruminantium, Appl. Environ. Microbiol. 63 (1997) 4853–4858. http://aem.asm.org/content/63/12/4853.full.pdf (accessed March 5, 2017).

[67] J.M. Lang, E.M. Benbouw, Species Interactions and Competition, Nat. Educ. Knowl. 4 (2013) 8.

[68] P. Mosoni, G. Fonty, P. Gouet, Competition Between Ruminal Cellulolytic Bacteria for Adhesion to Cellulose, Curr. Microbiol. 35 (1997) 44–47. doi:10.1007/s002849900209.

[69] Y. Shi, C.L. Odt, P.J. Weimer, Competition for Cellulose among Three Predominant Ruminal Cellulolytic Bacteria under Substrate-Excess and Substrate-Limited Conditions, Appl. Environ. Microbiol. 63 (1997) 734–742. http://aem.asm.org/content/63/2/734.full.pdf (accessed March 13, 2017).

[70] S.-C. Yang, C.-H. Lin, C.T. Sung, J.-Y. Fang, Antibacterial activities of bacteriocins: application in foods and pharmaceuticals., Front. Microbiol. 5 (2014) 241. doi:10.3389/fmicb.2014.00241.

[71] M.F. Whitford, M.A. McPherson, R.J. Forster, R.M. Teather, Identification of bacteriocin-like inhibitors from rumen Streptococcus spp. and isolation and characterization of bovicin 255., Appl. Environ. Microbiol. 67 (2001) 569–74. doi:10.1128/AEM.67.2.569-574.2001.

[72] A.C. Azevedo, C.B.P. Bento, J.C. Ruiz, M. V Queiroz, H.C. Mantovani, Distribution and Genetic Diversity of Bacteriocin Gene Clusters in Rumen Microbial Genomes., Appl. Environ. Microbiol. 81 (2015) 7290–304. doi:10.1128/AEM.01223-15.

[73] F. Li, G. Henderson, X. Sun, F. Cox, P.H. Janssen, L.L. Guan, Taxonomic Assessment of Rumen Microbiota Using Total RNA and Targeted Amplicon Sequencing Approaches., Front. Microbiol. 7 (2016) 987. doi:10.3389/fmicb.2016.00987.

[74] M.S. McCabe, P. Cormican, K. Keogh, A. O'Connor, E. O'Hara, R.A. Palladino, D.A. Kenny, S.M. Waters, Illumina MiSeq Phylogenetic Amplicon Sequencing Shows a Large Reduction of an Uncharacterised Succinivibrionaceae and an Increase of the Methanobrevibacter gottschalkii Clade in Feed Restricted Cattle, PLoS One. 10 (2015) e0133234. doi:10.1371/journal.pone.0133234.

[75] P.R. Myer, M. Kim, H.C. Freetly, T.P.L. Smith, Evaluation of 16S rRNA amplicon sequencing using two next-generation sequencing technologies for phylogenetic analysis of the rumen bacterial community in steers, J. Microbiol. Methods. 127 (2016) 132–140. doi:10.1016/j.mimet.2016.06.004.

[76] H.A. Paz, C.L. Anderson, M.J. Muller, P.J. Kononoff, S.C. Fernando, Rumen bacterial community composition in holstein and jersey cows is different under same dietary condition and is not affected by sampling method, Front. Microbiol. 7 (2016) 1–9. doi:10.3389/fmicb.2016.01206.

[77] C.J. Creevey, W.J. Kelly, G. Henderson, S.C. Leahy, Determining the culturability of the rumen bacterial microbiome., Microb. Biotechnol. 7 (2014) 467–79. doi:10.1111/1751-7915.12141.

[78] P.R. Myer, T.P.L. Smith, J.E. Wells, L.A. Kuehn, H.C. Freetly, G. Schroth, Rumen Microbiome from Steers Differing in Feed Efficiency, PLoS One. 10 (2015) e0129174. doi:10.1371/journal.pone.0129174.

[79] Y.-B. Chen, D.-L. Lan, C. Tang, X.-N. Yang, J. Li, Effect of DNA Extraction Methods on the Apparent Structure of Yak Rumen Microbial Communities as Revealed by 16S rDNA Sequencing., Polish J. Microbiol. 64 (2015) 29–36.

[80] R.J. Gruninger, C.W. Sensen, T.A. McAllister, R.J. Forster, Diversity of rumen bacteria in canadian cervids., PLoS One. 9 (2014) e89682. doi:10.1371/journal.pone.0089682.

[81] Rumen Microbial Genomics Network, (n.d.). http://www.rmgnetwork.org/home.html (accessed July 20, 2017).

[82] D. Hamar, R. Borchers, Glycolytic Pathway in Rumen Microorganisms, J. Anim. Sci. 26 (1967) 654. doi:10.2527/jas1967.263654x.

[83] E.A. Bayer, H. Chanzy, R. Lamed, Y. Shoham, Cellulose, cellulases and cellulosomes, Curr. Opin. Struct. Biol. 8 (1998) 548–557. doi:10.1016/S0959-440X(98)80143-7.

[84] L. Bao, Q. Huang, L. Chang, J. Zhou, H. Lu, Screening and characterization of a cellulase with endocellulase and exocellulase activity from yak rumen metagenome, J. Mol. Catal. B Enzym. 73 (2011) 104–110. doi:10.1016/j.molcatb.2011.08.006.

[85] M.J. Wolin, W.M. Regan, S.W. Mead, S. Lakshamanan, R.N. Doetsch, A Theoretical Rumen Fermentation Balance, J. Dairy Sci. 43 (1960) 1452–1459. doi:10.3168/jds.S0022-0302(60)90348-9.

[86] R. Wallace, The proteolytic systems of ruminal microorganisms, INRA/EDP Sci. 45 (1996) 301–308. https://hal.archives-ouvertes.fr/hal-00889635 (accessed August 18, 2017).

[87] F. Husvéth, Microbial digestion in the forestomachs of ruminants, in: Physiol. Reprod. Asp. Anim. Prod., 2011.

[88] J.W. Joo, G.S. Bae, W.K. Min, H.S. Choi, W.J. Maeng, Y.H. Chung, M.B. Chang, Effect of Protein Sources on Rumen Microbial Protein Synthesis Using Rumen Simulated Continuous Culture System, Asian-Australasian J. Anim. Sci. 18 (2005) 326–331. doi:10.5713/ajas.2005.326.

[89] B. Norton, R. Murray, K. Entwistle, J. Nolan, F. Ball, R. Leng, The nitrogen metabolism of sheep consuming Flinders grass (*Iseilema* spp.), Mitchell grass (*Astrebla* spp.) and mixed native pasture, Aust. J. Agric. Res. 29 (1978) 595. doi:10.1071/AR9780595.

[90] A. Sarraseca, E. Milne, M.J. Metcalf, G.E. Lobley, Urea recycling in sheep: effects of intake., Br. J. Nutr. 79 (1998) 79–88. http://www.ncbi.nlm.nih.gov/pubmed/9505805 (accessed November 23, 2017).

[91] G.E. Lobley, D.M. Bremner, G. Zuur, Effects of diet quality on urea fates in sheep as assessed by refined, non-invasive [15N15N]urea kinetics., Br. J. Nutr. 84 (2000) 459–68. http://www.ncbi.nlm.nih.gov/pubmed/11103216 (accessed November 23, 2017).

[92] H. Lapierre, G.E. Lobley, A.G. Calder, E. Milne, R.L. Coop, F. Jackson, G.W. Horgan, J.C. MacRae, C. Persaud, A. Jackson, Nitrogen Recycling in the Ruminant: A Review, J. Dairy Sci. 84 (2001) E223–E236. doi:10.3168/jds.S0022-0302(01)70222-6.

[93] A. Bach, S. Calsamiglia, M.D. Stern, Nitrogen Metabolism in the Rumen, J. Dairy Sci. 88 (2005) E9–E21. doi:10.3168/jds.S0022-0302(05)73133-7.

[94] G. Attwood, D. Li, D. Pacheco, M. Tavendale, Production of indolic compounds by rumen bacteria isolated from grazing ruminants, J. Appl. Microbiol. 100 (2006) 1261–1271. doi:10.1111/j.1365-2672.2006.02896.x.

[95] R. Zhang, W. Zhu, W. Zhu, J. Liu, S. Mao, Effect of dietary forage sources on rumen microbiota, rumen fermentation and biogenic amines in dairy cows, J. Sci. Food Agric. 94 (2014) 1886–1895. doi:10.1002/jsfa.6508.

[96] H. Wu, Q. Meng, Z. Yu, Effect of pH buffering capacity and sources of dietary sulfur on rumen fermentation, sulfide production, methane production, sulfate reducing bacteria, and total Archaea in in vitro rumen cultures, Bioresour. Technol. 186 (2015) 25–33. doi:10.1016/j.biortech.2015.02.110.

[97] A. Belanche, G. de la Fuente, C.J. Newbold, Study of methanogen communities associated with different rumen protozoal populations., FEMS Microbiol. Ecol. 90 (2014) 663–77. doi:10.1111/1574-6941.12423.

[98] P.H. Janssen, M. Kirs, Structure of the Archaeal Community of the Rumen, Appl. Environ. Microbiol. 74 (2008) 3619–3625. doi:10.1128/AEM.02812-07.

[99] K.D. Ashby, T.A. Casey, M.A. Rasmussen, J.W. Petrich, Steady-state and time-resolved spectroscopy of F420 extracted from methanogen cells and its utility as a marker for fecal contamination., J. Agric. Food Chem. 49 (2001) 1123–7.

[100] W.E. Balch, R.S. Wolfe, Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid)., J. Bacteriol. 137 (1979) 256–63.

[101] S.C. Leahy, W.J. Kelly, E. Altermann, R.S. Ronimus, C.J. Yeoman, D.M. Pacheco, D. Li, Z. Kong, S. McTavish, C. Sang, S.C. Lambie, P.H. Janssen, D. Dey, G.T. Attwood, The Genome Sequence of the Rumen Methanogen Methanobrevibacter ruminantium Reveals New Possibilities for Controlling Ruminant Methane Emissions, PLoS One. 5 (2010) e8926. doi:10.1371/journal.pone.0008926.

[102] R. Danielsson, J. Dicksved, L. Sun, H. Gonda, B. Müller, A. Schnürer, J. Bertilsson, Methane Production in Dairy Cows Correlates with Rumen Methanogenic and Bacterial Community Structure., Front. Microbiol. 8 (2017) 226. doi:10.3389/fmicb.2017.00226.

[103] G. Henderson, F. Cox, S. Ganesh, A. Jonker, W. Young, Global Rumen Consensus Collaborators, P.H. Janssen, Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range, Sci. Rep. 5 (2015) 14567. doi:10.1038/srep14567.

[104] T. Iino, H. Tamaki, S. Tamazawa, Y. Ueno, M. Ohkuma, K.-I. Suzuki, Y. Igarashi, S. Haruta, Candidatus Methanogranum caenicola: a novel methanogen from the anaerobic digested sludge, and proposal of Methanomassiliicoccaceae fam. nov. and Methanomassiliicoccales ord. nov., for a methanogenic lineage of the class Thermoplasmata., Microbes Environ. 28 (2013) 244–50.

[105] H. Seedorf, S. Kittelmann, G. Henderson, P.H. Janssen, RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments., PeerJ. 2 (2014) e494. doi:10.7717/peerj.494.

[106] W. Jin, Y. Cheng, S. Mao, W. Zhu, Discovery of a novel rumen methanogen in the anaerobic fungal culture and its distribution in the rumen as revealed by real-time PCR, BMC Microbiol. 14 (2014) 104. doi:10.1186/1471-2180-14-104.

[107] M. Poulsen, C. Schwab, B.B. Jensen, R.M. Engberg, A. Spang, N. Canibe, O. Højberg, G. Milinovich, L. Fragner, C. Schleper, W. Weckwerth, P. Lund, A. Schramm, T. Urich, Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen., Nat. Commun. 4 (2013) 1428. doi:10.1038/ncomms2432.

[108] C.A. Carberry, D.A. Kenny, A.K. Kelly, S.M. Waters, Quantitative analysis of ruminal methanogenic microbial populations in beef cattle divergent in phenotypic residual feed intake (RFI) offered contrasting diets., J. Anim. Sci. Biotechnol. 5 (2014) 41. doi:10.1186/2049-1891-5-41.

[109] X.D. Huang, G. Martinez-Fernandez, J. Padmanabha, R. Long, S.E. Denman, C.S. McSweeney, X.D. Huang, G. Martinez-Fernandez, J. Padmanabha, R. Long, S.E. Denman, C.S. McSweeney, Methanogen Diversity in Indigenous and Introduced Ruminant Species on the Tibetan Plateau, Archaea. 2016 (2016) 1–10. doi:10.1155/2016/5916067.

[110] H. Seedorf, S. Kittelmann, P.H. Janssen, Few highly abundant operational taxonomic units dominate within rumen methanogenic archaeal species in New Zealand sheep and cattle., Appl. Environ. Microbiol. 81 (2015) 986–95. doi:10.1128/AEM.03018-14.

[111] R.A. Leng, Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation, Anim. Prod. Sci. 54 (2014) 519. doi:10.1071/AN13381.

[112] M.J. Bell, S.L. Potterton, J. Craigon, N. Saunders, R.H. Wilcox, M. Hunter, J.R. Goodman, P.C. Garnsworthy, Variation in enteric methane emissions among cows on commercial dairy farms, Animal. 8 (2014) 1540–1546. doi:10.1017/S1751731114001530.

[113] Y. Dini, J. Gere, C. Briano, M. Manetti, P. Juliarena, V. Picasso, R. Gratton, L. Astigarraga, Methane Emission and Milk Production of Dairy Cows Grazing Pastures Rich in Legumes or Rich in Grasses in Uruguay, Animals. 2 (2012) 288–300. doi:10.3390/ani2020288.

[114] P.C. Garnsworthy, J. Craigon, J.H. Hernandez-Medrano, N. Saunders, Variation among individual dairy cows in methane measurements made on farm during milking, J. Dairy Sci. 95 (2012) 3181–3189. doi:10.3168/jds.2011-4606.

[115] R. Roehe, R.J. Dewhurst, C.-A. Duthie, J.A. Rooke, N. McKain, D.W. Ross, J.J. Hyslop, A. Waterhouse, T.C. Freeman, M. Watson, R.J. Wallace, Bovine Host Genetic Variation Influences Rumen Microbial Methane Production with Best Selection Criterion for Low Methane Emitting and Efficiently Feed Converting Hosts Based on Metagenomic Gene Abundance., PLoS Genet. 12 (2016) e1005846. doi:10.1371/journal.pgen.1005846.

[116] W.B. Shi, C.D. Moon, S.C. Leahy, D.W. Kang, J. Froula, S. Kittelmann, C. Fan, S. Deutsch, D. Gagic, H. Seedorf, W.J. Kelly, R. Atua, C. Sang, P. Soni, D. Li, C.S. Pinares-Patino, J.C. Mcewan, P.H. Janssen, F. Chen, A. Visel, Z. Wang, G.T. Attwood, E.M. Rubin, Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome, Genome Res. 24 (2014) 1517–1525. wos:000341281200010.

[117] https://tu-dresden.de/mn/biologie/mikro/mikdiv/forschung/Projects/methanogenesis (accessed March 19, 2018).

[118] K.J. Cheng, J.P. Fay, R.N. Coleman, L.P. Milligan, J.W. Costerton, Formation of bacterial microcolonies on feed particles in the rumen., Appl. Environ. Microbiol. 41 (1981) 298–305.

[119] W.M. Craig, G.A. Broderick, D.B. Ricker, Quantitation of microorganisms associated with the

particulate phase of ruminal ingesta., J. Nutr. 117 (1987) 56–62.

[120] C.A. Rodriguez, J. González, M.R. Alvir, R. Redondo, C. Cajarville, Effects of feed intake on composition of sheep rumen contents and their microbial population size, Br. J. Nutr. 89 (2003) 97. doi:10.1079/BJN2002752.

[121] A.G. Williams, N.H. Strachan, Polysaccharide degrading enzymes in microbial populations from the liquid and solid fractions of bovine rumen digesta, Can. J. Anim. Sci. 64 (1984) 58–59. doi:10.4141/cjas84-156.

[122] B. Michalet-Doreau, I. Fernandez, C. Peyron, L. Millet, G. Fonty, Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents, Reprod. Nutr. Dev. EDP Sci. 41 (2001) 187–194. doi:10.1051/rnd:2001122>.

[123] F.M. Brock, C.W. Forsberg, J.G. Buchanan-Smith2, Proteolytic Activity of Rumen Microorganisms and Effects of Proteinase Inhibitors, Appl. Environ. Microbiol. 44 (1982) 561–569.

[124] T.A. McAllister, H.D. Bae, G.A. Jones, K.J. Cheng, Microbial attachment and feed digestion in the rumen., J. Anim. Sci. 72 (1994) 3004–18.

[125] R. Larue, Z. Yu, V.A. Parisi, A.R. Egan, M. Morrison, Novel microbial diversity adherent to plant biomass in the herbivore gastrointestinal tract, as revealed by ribosomal intergenic spacer analysis and rrs gene sequencing, Environ. Microbiol. 7 (2005) 530–543. doi:10.1111/j.1462-2920.2005.00721.x.

[126] R.A. Leng, The Rumen - a fermentation vat or a series of organized structured microbial consortia: implications for the mitigation of enteric methane production by feed additives., Livest. Res. Rural Dev. 23 (2011).

[127] K. Cheng, R. McCowan, J. Costerton, Adherent epithelial bacteria in ruminants and their roles in digestive tract function, Am J Clin Nutr. 32 (1979) 139–148.

[128] K.J. Cheng, R.J. Wallace, The mechanism of passage of endogenous urea through the rumen wall and the role of ureolytic epithelial bacteria in the urea flux, Br. J. Nutr. 42 (1979) 553. doi:10.1079/BJN19790147.

[129] D. Dinsdale, K.J. Cheng, R.J. Wallace, R. a Goodlad, Digestion of epithelial tissue of the rumen wall by adherent bacteria in infused and conventionally fed sheep., Appl. Environ. Microbiol. 39 (1980) 1059–66.

[130] R.P. McCowan, K.J. Cheng, J.W. Costerton, Adherent bacterial populations on the bovine rumen wall: distribution patterns of adherent bacteria., Appl. Environ. Microbiol. 39 (1980) 233–41.

[131] H. Song, W.P. Clarke, L.L. Blackall, Concurrent microscopic observations and activity measurements of cellulose hydrolyzing and methanogenic populations during the batch anaerobic digestion of crystalline cellulose, Biotechnol. Bioeng. 91 (2005) 369–378. doi:10.1002/bit.20517.

[132] B.J. Finlay, G. Esteban, K.J. Clarke, A.G. Williams, T.M. Embley, R.P. Hirt, Some rumen ciliates have endosymbiotic methanogens., FEMS Microbiol. Lett. 117 (1994) 157–61.

[133] C.J. Newbold, B. Lassalas, J.P. Jouany, The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro, Lett. Appl. Microbiol. 21 (1995) 230–234. doi:10.1111/j.1472-765X.1995.tb01048.x.

[134] E. Jami, A. Israel, A. Kotser, I. Mizrahi, Exploring the bovine rumen bacterial community from birth to adulthood, ISME J. 7 (2013) 1069–1079. doi:10.1038/ismej.2013.2.

[135] D.R. Yáñez-Ruiz, L. Abecia, C.J. Newbold, Manipulating rumen microbiome and fermentation through interventions during early life: a review., Front. Microbiol. 6 (2015) 1133. doi:10.3389/fmicb.2015.01133.

[136] J. Hernández, J.L. Benedito, A. Abuelo, C. Castillo, Luis, A. Abuelo, C. Castillo, Ruminal acidosis in feedlot: from aetiology to prevention., ScientificWorldJournal. 2014 (2014) 702572. doi:10.1155/2014/702572.

[137] I.S. Kim, M.H. Hwang, N.J. Jang, S.H. Hyun, S.T. Lee, Effect of low pH on the activity of hydrogen utilizing methanogen in bio-hydrogen process, Int. J. Hydrogen Energy. 29 (2004) 1133–1140. doi:10.1016/j.ijhydene.2003.08.017.

[138] J.A.Z. Leedle, K. Barsuhn, R.B. Hespell, Postprandial Trends in Estimated Ruminal Digesta Polysaccharides and Their Relation to Changes in Bacterial Groups and Ruminal Fluid Characteristics, J. Anim. Sci. 62 (1986) 789. doi:10.2527/jas1986.623789x.

[139] F. Mouriño, R. Akkarawongsa, P.J. Weimer, Initial pH as a Determinant of Cellulose Digestion Rate by Mixed Ruminal Microorganisms In Vitro, J. Dairy Sci. 84 (2001) 848–859. doi:10.3168/jds.S0022-0302(01)74543-2.

[140] Final Report Summary - RUMINOMICS (Connecting the animal genome, gastrointestinal microbiomes and nutrition to improve digestion efficiency and the environmental impacts of ruminant livestock production), (n.d.). http://cordis.europa.eu/result/rcn/191776_en.html (accessed February 24, 2017).

[141] K.A. Beauchemin, S.M. McGinn, Enteric methane emissions from growing beef cattle as affected by diet and level of intake, Can. J. Anim. Sci. 86 (2006) 401–408. doi:10.4141/A06-021.

[142] P.A. Foley, D.A. Kenny, J.J. Callan, T.M. Boland, F.P. O'Mara, Effect of DL-malic acid supplementation on feed intake, methane emission, and rumen fermentation in beef cattle., J. Anim. Sci. 87 (2009) 1048–57. doi:10.2527/jas.2008-1026.

[143] R.R. Hofmann, Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system, Oecologia. 78 (1989) 443–457. doi:10.1007/BF00378733.

[144] M. Beecher, F. Buckley, S.M. Waters, T.M. Boland, D. Enriquez-Hidalgo, M.H. Deighton, M. O'Donovan, E. Lewis, Gastrointestinal tract size, total-tract digestibility, and rumen microflora in different dairy cow genotypes, J. Dairy Sci. 97 (2014) 3906–3917. doi:10.3168/jds.2013-7708.

[145] J.A. Rooke, R.J. Wallace, C.-A. Duthie, N. McKain, S.M. de Souza, J.J. Hyslop, D.W. Ross, T. Waterhouse, R. Roehe, Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype, Br. J. Nutr. 112 (2014) 398–407. doi:10.1017/S0007114514000932.

[146] S.L. Woodward, G.C. Waghorn, M.J. Ulyatt, K.R. Lassey, Early indications that feeding Lotus will reduce methane emissions from ruminants, in: Proc. New Zeal. Soc. Anim. Prod., 2001: pp. 23–26.

[147] J.B. Vlaming, N. Lopez-Villalobos, I.M. Brookes, S.O. Hoskin, H. Clark, Within- and between-animal variance in methane emissions in non-lactating dairy cows, Aust. J. Exp. Agric. 48 (2008) 124. doi:10.1071/EA07278.

[148] E. Hernandez-Sanabria, L.L. Guan, L.A. Goonewardene, M. Li, D.F. Mujibi, P. Stothard, S.S. Moore, M.C. Leon-Quintero, Correlation of particular bacterial PCR-denaturing gradient gel electrophoresis patterns with bovine ruminal fermentation parameters and feed efficiency traits., Appl. Environ. Microbiol. 76 (2010) 6338–50. doi:10.1128/AEM.01052-10.

[149] E. Jami, I. Mizrahi, F. Bushman, T. DeSantis, G. Andersen, Composition and Similarity of Bovine Rumen Microbiota across Individual Animals, PLoS One. 7 (2012) e33306. doi:10.1371/journal.pone.0033306.

[150] P.R. Myer, T.P.L. Smith, J.E. Wells, L.A. Kuehn, H.C. Freetly, G. Schroth, Rumen Microbiome from Steers Differing in Feed Efficiency, PLoS One. 10 (2015) e0129174. doi:10.1371/journal.pone.0129174.

[151] P.J. Weimer, D.M. Stevenson, H.C. Mantovani, S.L.C. Man, Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents, J. Dairy Sci. 93 (2010) 5902–5912. wos:000284404400039.

[152] M. Ogle, Riots, Rage, and Resistance: A Brief History of How Antibiotics Arrived on the Farm, Sci. Am. (2013). https://blogs.scientificamerican.com/guest-blog/riots-rage-and-resistance-a-brief-history-of-how-antibiotics-arrived-on-the-farm/ (accessed March 31, 2017).

[153] EC, Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, (2003). http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32003R1831 (accessed March 31, 2017).

[154] FDA, New Animal Drugs for Use in Animal Feed; Approval of New Animal Drug Applications; Withdrawal of Approval of New Animal Drug Applications, (2016). https://www.federalregister.gov/documents/2016/12/27/2016-31083/new-animal-drugs-for-use-in-animal-feed-approval-of-new-animal-drug-applications-withdrawal-of (accessed March 31, 2017).

[155] M. Mellon, C. Benbrook, K. Lutz Benbrook, Hogging It Estimates of Antimicrobial Abuse in Livestock, Union Concerned Sci. (2001). http://www.ucsusa.org/sites/default/files/legacy/assets/documents/food_and_agriculture/hog_front.pdf (accessed March 31, 2017).

[156] BelVet-SAC, Belgian Veterinary Surveillance of Antibacterial Consumption: annual report 2016, (2017). http://www.belvetsac.ugent.be/BelvetSAC_report_2016.pdf (accessed August 1, 2015).

[157] B. Callens, D. Persoons, D. Maes, M. Laanen, M. Postma, F. Boyen, F. Haesebrouck, P. Butaye, B. Catry, J. Dewulf, Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds, Prev. Vet. Med. 106 (2012) 53–62. wos:000307155000006.

[158] T.F. Landers, B. Cohen, T.E. Wittum, E.L. Larson, A review of antibiotic use in food animals: perspective, policy, and potential., Public Health Rep. 127 (2012) 4–22. doi:10.1177/003335491212700103.

[159] M.E. Filippitzi, S. Sarrazin, H. Imberechts, A. Smet, J. Dewulf, Risk of cross-contamination due to the use of antimicrobial medicated feed throughout the trail of feed from the feed mill to the farm., Food Addit. Contam. Part A. Chem. Anal. Control. Expo. Risk Assess. 33 (2016) 644–55. doi:10.1080/19440049.2016.1160442.

[160] A.A.M. Stolker, V. Manti, T. Zuidema, H. van Egmond, E.R. Deckers, R. Herbes, J. Hooglugt, E. Olde Heuvel, J. de Jong, Carry-over of veterinary drugs from medicated to non-medicated feeds in commercial feed manufacturing plants, Food Addit. Contam. Part A. 30 (2013) 1100–1107. doi:10.1080/19440049.2013.794308.

[161] T. Zuidema, A.A.M. Stolker, L.A. van Ginkel, Kruiscontaminatie van antibiotica. Onderzoek naar de aanwezigheid van antibioticaresiduen in mest van vleesvarkens en vleeskalveren en op pluimveebedrijven., RIKILT-Rapport. (2014).

[162] D. Schlessinger, Failure of aminoglycoside antibiotics to kill anaerobic, low-pH, and resistant

cultures., Clin. Microbiol. Rev. 1 (1988) 54–9.

[163] H.C. Neu, T.D. Gootz, Antimicrobial Chemotherapy, University of Texas Medical Branch at Galveston, 1996. http://www.ncbi.nlm.nih.gov/pubmed/21413283 (accessed April 3, 2017).

[164] S.M. Soucy, J. Huang, J.P. Gogarten, Horizontal gene transfer: building the web of life, Nat. Rev. Genet. 16 (2015) 472–482. doi:10.1038/nrg3962.

[165] M. Zatyka, C.M. Thomas, Control of genes for conjugative transfer of plasmids and other mobile elements, FEMS Microbiol. Rev. 21 (1998) 291–319. doi:10.1111/j.1574-6976.1998.tb00355.x.

[166] G. del Solar, R. Giraldo, M.J. Ruiz-Echevarría, M. Espinosa, R. Díaz-Orejas, Replication and control of circular bacterial plasmids., Microbiol. Mol. Biol. Rev. 62 (1998) 434–64.

[167] N.L. Craig, TARGET SITE SELECTION IN TRANSPOSITION, Annu. Rev. Biochem. 66 (1997) 437–474. doi:10.1146/annurev.biochem.66.1.437.

[168] R.P. Novick, Plasmid incompatibility., Microbiol. Rev. 51 (1987) 381–95. http://www.ncbi.nlm.nih.gov/pubmed/3325793 (accessed April 5, 2017).

[169] P.M. Bennett, Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria., Br. J. Pharmacol. (2008) S347-57. doi:10.1038/sj.bjp.0707607.

[170] C.I. Mortvedt, I.F. N E S, Plasmid-associated bacteriocin production by a LactobaciZZus sake strain, J. Gen. Microbiol. 136 (1990) 1601–1607.

[171] R.S. Burlage, S.W. Hooper, G.S. Sayler, The TOL (pWW0) catabolic plasmid., Appl. Environ. Microbiol. 55 (1989) 1323–8.

[172] J. Li, V. Adams, T.L. Bannam, K. Miyamoto, J.P. Garcia, F.A. Uzal, J.I. Rood, B.A. McClane, Toxin Plasmids of Clostridium perfringens, Microbiol. Mol. Biol. Rev. 77 (2013) 208–233. doi:10.1128/MMBR.00062-12.

[173] L.E. Peeters, El. Daeselaire, M. Devreese, G. Rasschaert, A. Smet, M. Heyndrickx, H. Imbrechts, F. Haesebrouck, P. Butaye, S. Croubels, Residues of Chlortetracycline, Doxycycline and Sulfadiazine-Trimethoprim in Intestinal Content and Feces of Pigs due to Cross-contamination of Feed, BMC Vet. Res. 12 (2016) 209.

[174] B.M. Marshall, S.B. Levy, Food animals and antimicrobials: impacts on human health., Clin. Microbiol. Rev. 24 (2011) 718–33. doi:10.1128/CMR.00002-11.

[175] T. Looft, T.A. Johnson, H.K. Allen, D.O. Bayles, D.P. Alt, R.D. Stedtfeld, W.J. Sul, T.M. Stedtfeld, B.L. Chai, J.R. Cole, S.A. Hashsham, J.M. Tiedje, T.B. Stanton, In-feed antibiotic effects on the swine intestinal microbiome, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 1691–1696. wos:000299731400069.

[176] H. Bum Kim, K. Borewicz, B.A. White, R.S. Singer, S. Sreevatsan, Z. Jin Tu, R.E. Isaacson, by W. Harley Moon, Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin, (n.d.). doi:10.1073/pnas.1205147109.

[177] D.B. Holman, M.R. Chénier, Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine, FEMS Microbiol. Ecol. 90 (2014) 599–608. doi:10.1111/1574-6941.12419.

[178] S. Reardon, Spread of antibiotic-resistance gene does not spell bacterial apocalypse — yet, Nature. (2015). doi:10.1038/nature.2015.19037.

[179] A.E.J.M. van den Bogaard, N. London, E.E. Stobberingh, Antimicrobial resistance in pig faecal

samples from The Netherlands (five abattoirs) and Sweden, J. Antimicrob. Chemother. 45 (2000) 663–671. doi:10.1093/jac/45.5.663.

[180] Y.-Y. Liu, Y. Wang, T.R. Walsh, L.-X. Yi, R. Zhang, J. Spencer, Y. Doi, G. Tian, B. Dong, X. Huang, L.-F. Yu, D. Gu, H. Ren, X. Chen, L. Lv, D. He, H. Zhou, Z. Liang, J.-H. Liu, J. Shen, Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study, Lancet Infect. Dis. 16 (2016) 161–168. doi:10.1016/S1473-3099(15)00424-7.

[181] N.T. Nguyen, H.M. Nguyen, C. V Nguyen, T. V Nguyen, M.T. Nguyen, H.Q. Thai, M.H. Ho, G. Thwaites, H.T. Ngo, S. Baker, J. Carrique-Mas, Use of Colistin and Other Critical Antimicrobials on Pig and Chicken Farms in Southern Vietnam and Its Association with Resistance in Commensal Escherichia coli Bacteria., Appl. Environ. Microbiol. 82 (2016) 3727–35. doi:10.1128/AEM.00337-16.

[182] A.O. Olaitan, S. Chabou, L. Okdah, S. Morand, J.-M. Rolain, Dissemination of the mcr-1 colistin resistance gene., Lancet. Infect. Dis. 16 (2016) 147. doi:10.1016/S1473-3099(15)00540-X.

[183] H.E. Webb, S.A. Granier, M. Marault, Y. Millemann, H.C. den Bakker, K.K. Nightingale, M. Bugarel, S.A. Ison, H.M. Scott, G.H. Loneragan, Dissemination of the mcr-1 colistin resistance gene., Lancet. Infect. Dis. 16 (2016) 144–5. doi:10.1016/S1473-3099(15)00538-1.

[184] M.S. Arcilla, J.M. van Hattem, S. Matamoros, D.C. Melles, J. Penders, M.D. de Jong, C. Schultsz, Dissemination of the mcr-1 colistin resistance gene, Lancet Infect. Dis. 16 (2016) 147–149. doi:10.1016/S1473-3099(15)00541-1.

[185] H. Hasman, A.M. Hammerum, F. Hansen, R.S. Hendriksen, B. Olesen, Y. Agersø, E. Zankari, P. Leekitcharoenphon, M. Stegger, R.S. Kaas, L.M. Cavaco, D.S. Hansen, F.M. Aarestrup, R.L. Skov, Detection of *mcr-1* encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015, Eurosurveillance. 20 (2015) 30085. doi:10.2807/1560-7917.ES.2015.20.49.30085.

[186] W. Yin, H. Li, Y. Shen, Z. Liu, S. Wang, Z. Shen, R. Zhang, T.R. Walsh, J. Shen, Y. Wang, Novel Plasmid-Mediated Colistin Resistance Gene mcr-3 in Escherichia coli., MBio. 8 (2017) e00543-17. doi:10.1128/mBio.00543-17.

[187] S.B. LEVY, G.B. FITZGERALD, A.B. MACONE, Spread of antibiotic-resistant plasmids from chicken to chicken and from chicken to man, Nature. 260 (1976) 40–42. doi:10.1038/260040a0.

[188] H. Aubry-Damon, K. Grenet, P. Sall-Ndiaye, D. Che, E. Cordeiro, M.-E. Bougnoux, E. Rigaud, Y. Le Strat, V. Lemanissier, L. Armand-Lefèvre, D. Delzescaux, J.-C. Desenclos, M. Liénard, A. Andremont, Antimicrobial resistance in commensal flora of pig farmers., Emerg. Infect. Dis. 10 (2004) 873–9. doi:10.3201/eid1005.030735.

[189] T.W. Alexander, G.D. Inglis, L.J. Yanke, E. Topp, R.R. Read, T. Reuter, T.A. McAllister, Farm-to-fork characterization of Escherichia coli associated with feedlot cattle with a known history of antimicrobial use, Int. J. Food Microbiol. 137 (2010) 40–48. doi:10.1016/j.ijfoodmicro.2009.11.008.

[190] E. de Boer, J.T.M. Zwartkruis-Nahuis, B. Wit, X.W. Huijsdens, A.J. de Neeling, T. Bosch, R.A.A. van Oosterom, A. Vila, A.E. Heuvelink, Prevalence of methicillin-resistant Staphylococcus aureus in meat, Int. J. Food Microbiol. 134 (2009) 52–56. doi:10.1016/j.ijfoodmicro.2008.12.007.

[191] M.E. Doyle, Multidrug-Resistant Pathogens in the Food Supply, Foodborne Pathog. Dis. 12 (2015) 261–279. doi:10.1089/fpd.2014.1865.

[192] K.E. Smith, J.M. Besser, C.W. Hedberg, F.T. Leano, J.B. Bender, J.H. Wicklund, B.P. Johnson,

K.A. Moore, M.T. Osterholm, Quinolone-Resistant *Campylobacter jejuni* Infections in Minnesota, 1992–1998, N. Engl. J. Med. 340 (1999) 1525–1532. doi:10.1056/NEJM199905203402001.

[193] CDC, Multistate Outbreak of Multidrug-Resistant Salmonella I 4,[5],12:i:- and Salmonella infantis Infections Linked to Pork (Final Update), (2015). https://www.cdc.gov/salmonella/pork-08-15/ (accessed April 10, 2017).

[194] S.A. McEwen, R. Reid-Smith, Antimicrobial resistance in food., Can. J. Infect. Dis. Med. Microbiol. = J. Can. Des Mal. Infect. La Microbiol. Medicale. 15 (2004) 201–3.

[195] R. Marti, A. Scott, Y.-C. Tien, R. Murray, L. Sabourin, Y. Zhang, E. Topp, Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest., Appl. Environ. Microbiol. 79 (2013) 5701–9. doi:10.1128/AEM.01682-13.

[196] A.A. Salyers, C.F. Amábile-Cuevas, Why are antibiotic resistance genes so resistant to elimination?, Antimicrob. Agents Chemother. 41 (1997) 2321–5.

[197] M. Tamminen, A. Karkman, A. Lõhmus, W.I. Muziasari, H. Takasu, S. Wada, S. Suzuki, M. Virta, Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure., Environ. Sci. Technol. 45 (2011) 386–91. doi:10.1021/es102725n.

[198] EMA, Sales of veterinary antimicrobial agents in 30 European countries in 2015, Seventh ESVAC Rep. (2017). http://www.ema.europa.eu/docs/en_GB/document_library/Report/2017/10/WC500236750.pdf (accessed April 10, 2017).

[199] J.J. Li, E.J. Corey, Drug discovery practices, processes, and perspectives, John Wiley & Sons, 2013. https://books.google.ca/books?id=mIyxO5cLEAcC&pg=PA406#v=onepage&q=doxycycline&f=false (accessed April 10, 2017).

[200] L. Gutiérrez, L. Ocampo, F. Espinosa, H. Sumano, Pharmacokinetics of an injectable long-acting parenteral formulation of doxycycline hyclate in pigs, J. Vet. Pharmacol. Ther. 37 (2014) 83–89. doi:10.1111/jvp.12066.

[201] K. Baert, S. Croubels, F. Gasthuys, J. De Busser, P. De Backer, Pharmacokinetics and oral bioavailability of a doxycycline formulation (DOXYCYCLINE 75%) in nonfasted young pigs, J. Vet. Pharmacol. Ther. 23 (2000) 45–48. doi:10.1046/j.1365-2885.2000.00235.x.

[202] J.R.E. Del Castillo, V. Laroute, P. Pommier, C. Zé, A. Keta, D. Concordet, P.-L. Toutain, Interindividual variability in plasma concentrations after systemic exposure of swine to dietary doxycycline supplied with and without paracetamol: A population pharmacokinetic approach 1, J. Anim. Sci. 84 (2006) 3155–3166. doi:10.2527/jas.2005-561.

[203] I. Chopra, M. Roberts, Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance, Microbiol. Mol. Biol. Rev. 65 (2001) 232–+. wos:000169135200003.

[204] M.C. Nelson, L. Bomar, M. Maltz, J. Graf, Mucinivorans hirudinis gen. nov., sp. nov., an anaerobic, mucin-degrading bacterium isolated from the digestive tract of the medicinal leech Hirudo verbana, Int. J. Syst. Evol. Microbiol. 65 (2015) 990–995. doi:10.1099/ijs.0.000052.

[205] A.E. Asagbra, A.I. Sanni, O.B. Oyewole, Solid-state fermentation production of tetracycline by Streptomyces strains using some agricultural wastes as substrate, World J. Microbiol. Biotechnol. 21 (2005) 107–114. doi:10.1007/s11274-004-2778-z.

[206] R.E. de Lima Procópio, I.R. da Silva, M.K. Martins, J.L. de Azevedo, J.M. de Araújo, Antibiotics produced by Streptomyces, Brazilian J. Infect. Dis. 16 (2012) 466–471. doi:10.1016/j.bjid.2012.08.014.

[207] V.M. D'Costa, C.E. King, L. Kalan, M. Morar, W.W.L. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels, R. Debruyne, G.B. Golding, H.N. Poinar, G.D. Wright, Antibiotic resistance is ancient, Nature. 477 (2011) 457–461. doi:10.1038/nature10388.

[208] J.I. Ross, E.A. Eady, J.H. Cove, W.J. Cunliffe, 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium., Antimicrob. Agents Chemother. 42 (1998) 1702–5.

[209] L. Nonaka, S.R. Connell, D.E. Taylor, 16S rRNA mutations that confer tetracycline resistance in Helicobacter pylori decrease drug binding in Escherichia coli ribosomes., J. Bacteriol. 187 (2005) 3708–12. doi:10.1128/JB.187.11.3708-3712.2005.

[210] A.H. Delcour, Outer membrane permeability and antibiotic resistance., Biochim. Biophys. Acta. 1794 (2009) 808–16. doi:10.1016/j.bbapap.2008.11.005.

[211] M.C. Roberts, Mechanisms of bacterial antibiotic resistance and lessons learned from environmental tetracycline-resistant bacteria, in: P.L. Keen, M.H.M.M. Montforts (Eds.), Antimicrob. Resist. Environ., Wiley-Blackwell, 2012: pp. 93–121.

[212] M.A. Webber, L.J. V. Piddock, The importance of efflux pumps in bacterial antibiotic resistance, J. Antimicrob. Chemother. 51 (2003) 9–11. doi:10.1093/jac/dkg050.

[213] J. Sun, Z. Deng, A. Yan, Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations, Biochem. Biophys. Res. Commun. 453 (2014) 254–267. doi:10.1016/j.bbrc.2014.05.090.

[214] K. Poole, Efflux-mediated antimicrobial resistance, J. Antimicrob. Chemother. 56 (2005) 20–51. doi:10.1093/jac/dki171.

[215] T.A. Krulwich, J. Jin, A.A. Guffanti, H. Bechhofer, Functions of tetracycline efflux proteins that do not involve tetracycline., J. Mol. Microbiol. Biotechnol. 3 (2001) 237–46.

[216] A. Yamaguchi, Y. Shiina, E. Fujihira, T. Sawai, N. Noguchi, M. Sasatsu, The tetracycline efflux protein encoded by the *tet* (K) gene from *Staphylococcus aureus* is a metal-tetracycline/H^+ antiporter, FEBS Lett. 365 (1995) 193–197. doi:10.1016/0014-5793(95)00455-I.

[217] S.R. Connell, D.M. Tracz, K.H. Nierhaus, D.E. Taylor, Ribosomal protection proteins and their mechanism of tetracycline resistance., Antimicrob. Agents Chemother. 47 (2003) 3675–81. doi:10.1128/AAC.47.12.3675-3681.2003.

[218] M.C. Roberts, S. Schwarz, Tetracycline and Phenicol Resistance Genes and Mechanisms: Importance for Agriculture, the Environment, and Humans, J. Environ. Qual. 45 (2016) 576. doi:10.2134/jeq2015.04.0207.

[219] R.I. Aminov, J.C. Chee-Sanford, N. Garrigues, B. Teferedegne, I.J. Krapac, B.A. White, R.I. Mackie, Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria., Appl. Environ. Microbiol. 68 (2002) 1786–93. doi:10.1128/aem.68.4.1786-1793.2002.

[220] R.I. Aminov, R.I. Mackie, Evolution and ecology of antibiotic resistance genes, FEMS Microbiol. Lett. 271 (2007) 147–161. doi:10.1111/j.1574-6968.2007.00757.x.

[221] L. Nonaka, K. Ikeno, S. Suzuki, Distribution of Tetracycline Resistance Gene, tet(M), in Gram-Positive and Gram-Negative Bacteria Isolated from Sediment and Seawater at a Coastal Aquaculture Site in Japan, Microbes Environ. 22 (2007) 355–364.

http://ci.nii.ac.jp/naid/110006473362/en (accessed April 12, 2017).

[222] K.K. Ojo, D. Tung, H. Luis, M. Bernardo, J. Leitao, M.C. Roberts, Gram-positive *merA* gene in gram-negative oral and urine bacteria, FEMS Microbiol. Lett. 238 (2004) 411–416. doi:10.1111/j.1574-6968.2004.tb09783.x.

[223] M.C. Roberts, Resistance to Tetracycline,Macrolide-Lincosamide-Streptogramin, Trimethoprim,and Sulfonamide Drug Classes, Mol. Biotechnol. 20 (2002) 261–284. doi:10.1385/MB:20:3:261.

[224] W.E. Bentley, N. Mirjalili, D.C. Andersen, R.H. Davis, D.S. Kompala, Plasmid-encoded protein: The principal factor in the "metabolic burden" associated with recombinant bacteria, Biotechnol. Bioeng. 35 (1990) 668–681. doi:10.1002/bit.260350704.

[225] M. Rysz, W.R. Mansfield, J.D. Fortner, P.J.J. Alvarez, Tetracycline Resistance Gene Maintenance under Varying Bacterial Growth Rate, Substrate and Oxygen Availability, and Tetracycline Concentration, Environ. Sci. Technol. (2013) 130205085536002. doi:10.1021/es3035329.

[226] U. Zielenkiewicz, P. Cegłowski, Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems., Acta Biochim. Pol. 48 (2001) 1003–23.

[227] J.S. Shen, Z. Chai, L.J. Song, J.X. Liu, Y.M. Wu, Insertion depth of oral stomach tubes may affect the fermentation parameters of ruminal fluid collected in dairy cows, J. Dairy Sci. 95 (2012) 5978–5984. doi:10.3168/jds.2012-5499.

[228] I. Tapio, K.J. Shingfield, N. McKain, A. Bonin, D. Fischer, A.R. Bayat, J. Vilkki, P. Taberlet, T.J. Snelling, R.J. Wallace, Oral Samples as Non-Invasive Proxies for Assessing the Composition of the Rumen Microbial Community, PLoS One. 11 (2016) e0151220. doi:10.1371/journal.pone.0151220.

[229] J.W. Czerkawski, G. Breckenridge, Design and development of a long-term rumen simulation technique (Rusitec)., Br. J. Nutr. 38 (1977) 371–84. http://www.ncbi.nlm.nih.gov/pubmed/588537 (accessed November 23, 2017).

[230] A. Belanche, E. Jones, I. Parveen, C.J. Newbold, A Metagenomics Approach to Evaluate the Impact of Dietary Supplementation with Ascophyllum nodosum or Laminaria digitata on Rumen Function in Rusitec Fermenters., Front. Microbiol. 7 (2016) 299. doi:10.3389/fmicb.2016.00299.

[231] J. Mi, J. Zhou, X. Huang, R. Long, Lower Methane Emissions from Yak Compared with Cattle in Rusitec Fermenters, PLoS One. 12 (2017) e0170044. doi:10.1371/journal.pone.0170044.

[232] M. Jacobson, J. Erik Lindberg, R. Lindberg, C. Hård af Segerstad, P. Wallgren, C. Fellström, C. Hultén, M. Jensen-Waern, Intestinal Cannulation: Model for Study of the Midgut of the Pig, (2001). http://www.ingentaconnect.com/content/aalas/cm/2001/00000051/00000002/art00011?crawler=true (accessed March 1, 2018).

[233] A. Schwiertz, ed., Microbiota of the Human Body, Springer International Publishing, Cham, 2016. doi:10.1007/978-3-319-31248-4.

[234] J.B. Hughes, J.J. Hellmann, T.H. Ricketts, B.J. Bohannan, Counting the uncountable: statistical approaches to estimating microbial diversity., Appl. Environ. Microbiol. 67 (2001) 4399–406. doi:10.1128/AEM.67.10.4399-4406.2001.

[235] R.H. Whittaker, Vegetation of the Siskiyou Mountains, Oregon and California, Ecol. Monogr. 30 (1960) 279–338. doi:10.2307/1943563.

[236] C. Lozupone, M.E. Lladser, D. Knights, J. Stombaugh, R. Knight, UniFrac: an effective distance metric for microbial community comparison., ISME J. 5 (2011) 169–72. doi:10.1038/ismej.2010.133.

[237] R.J. Williams, A. Howe, K.S. Hofmockel, Demonstrating microbial co-occurrence pattern analyses within and between ecosystems., Front. Microbiol. 5 (2014) 358. doi:10.3389/fmicb.2014.00358.

[238] E.N. Bergman, Energy contributions of volatile fatty acids from the gastrointestinal tract in various species., Physiol. Rev. 70 (1990) 567–90.

[239] E.P.J.G. Neis, C.H.C. Dejong, S.S. Rensen, The role of microbial amino acid metabolism in host metabolism., Nutrients. 7 (2015) 2930–46. doi:10.3390/nu7042930.

[240] E.J. Zarling, M.A. Ruchim, Protein origin of the volatile fatty acids isobutyrate and isovalerate in human stool., J. Lab. Clin. Med. 109 (1987) 566–70.

[241] S. De Campeneere, N. Peiren, ILVO's Ruminant Respiration Facility. Chapter 3 in Technical Manual on Respiration Chamber Design. Melle, Belgium, (2014) 43–57.

[242] P.C. Garnsworthy, J. Craigon, J.H. Hernandez-Medrano, N. Saunders, On-farm methane measurements during milking correlate with total methane production by individual dairy cows, J. Dairy Sci. 95 (2012) 3166–3180. doi:10.3168/jds.2011-4605.

[243] C. Grainger, T. Clarke, S.M. McGinn, M.J. Auldist, K.A. Beauchemin, M.C. Hannah, G.C. Waghorn, H. Clark, R.J. Eckard, Methane Emissions from Dairy Cows Measured Using the Sulfur Hexafluoride (SF6) Tracer and Chamber Techniques, J. Dairy Sci. 90 (2007) 2755–2766. doi:10.3168/jds.2006-697.

[244] T. McAllister, H. Bae, G. Jones, K. Cheng, Microbial attachment and feed digestion in the rumen., J. Anim. Sci. 72 (1994) 3004–3018. doi:/1994.72113004x.

[245] H. Piao, M. Lachman, S. Malfatti, A. Sczyrba, B. Knierim, M. Auer, S.G. Tringe, R.I. Mackie, C.J. Yeoman, M. Hess, Temporal dynamics of fibrolytic and methanogenic rumen microorganisms during in situ incubation of switchgrass determined by 16S rRNA gene profiling, Front. Microbiol. 5 (2014) 1–11. doi:10.3389/fmicb.2014.00307.

[246] C.A. Rodríguez, J. González, M.R. Alvir, J.L. Repetto, C. Centeno, F. Lamrani, Composition of bacteria harvested from the liquid and solid fractions of the rumen of sheep as influenced by feed intake., Br. J. Nutr. 84 (2000) 369–76.

[247] W. Huo, W. Zhu, S. Mao, Impact of subacute ruminal acidosis on the diversity of liquid and solid-associated bacteria in the rumen of goats, World J. Microbiol. Biotechnol. 30 (2014) 669–680. doi:10.1007/s11274-013-1489-8.

[248] K.A. Jewell, C.A. McCormick, C.L. Odt, P.J. Weimer, G. Suen, Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency., Appl. Environ. Microbiol. 81 (2015) 4697–710. doi:10.1128/AEM.00720-15.

[249] H. Makkar, C. McSweeney, Methods in Gut Microbial Ecology for Ruminants, in: Springer, 2005: pp. 85–86. doi:10.1007/1-4020-3791-0.

[250] Z. Yu, M. Morrison, Improved extraction of PCR-quality community DNA from digesta and fecal samples, Biotechniques. 36 (2004) 808–812. doi:10.2144/3605A0808.

[251] R. Vilchez-Vargas, R. Geffers, M. Suárez-Diez, I. Conte, A. Waliczek, V.S. Kaser, M. Kralova, H. Junca, D.H. Pieper, Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system.,

Environ. Microbiol. 15 (2013) 1016–39. doi:10.1111/j.1462-2920.2012.02752.x.

[252] Illumina, Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System, 2013.

[253] S. Kittelmann, H. Seedorf, W.A. Walters, J.C. Clemente, R. Knight, J.I. Gordon, P.H. Janssen, Simultaneous Amplicon Sequencing to Explore Co-Occurrence Patterns of Bacterial, Archaeal and Eukaryotic Microorganisms in Rumen Microbial Communities, PLoS One. 8 (2013) e47879. doi:10.1371/journal.pone.0047879.

[254] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data., Bioinformatics. 30 (2014) 2114–2120. doi:10.1093/bioinformatics/btu170.

[255] R.C. Edgar, Search and clustering orders of magnitude faster than BLAST., Bioinformatics. 26 (2010) 2460–1. doi:10.1093/bioinformatics/btq461.

[256] J. Zhang, K. Kobert, T. Flouri, A. Stamatakis, PEAR: a fast and accurate Illumina Paired-End reAd mergeR., Bioinformatics. 30 (2014) 614–20. doi:10.1093/bioinformatics/btt593.

[257] R.C. Edgar, UPARSE: highly accurate OTU sequences from microbial amplicon reads., Nat. Methods. 10 (2013) 996–8. doi:10.1038/nmeth.2604.

[258] R.C. Edgar, B.J. Haas, J.C. Clemente, C. Quince, R. Knight, UCHIME improves sensitivity and speed of chimera detection., Bioinformatics. 27 (2011) 2194–200. doi:10.1093/bioinformatics/btr381.

[259] D. McDonald, J.C. Clemente, J. Kuczynski, J. Rideout, J. Stombaugh, D. Wendel, A. Wilke, S. Huse, J. Hufnagle, F. Meyer, R. Knight, J. Caporaso, The Biological Observation Matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome, Gigascience. 1 (2012) 7. doi:10.1186/2047-217X-1-7.

[260] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Peña, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, R. Knight, QIIME allows analysis of high-throughput community sequencing data., Nat. Methods. 7 (2010) 335–6. doi:10.1038/nmeth.f.303.

[261] T.Z. DeSantis, P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, G.L. Andersen, Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB., Appl. Environ. Microbiol. 72 (2006) 5069–72. doi:10.1128/AEM.03006-05.

[262] J.G. Caporaso, K. Bittinger, F.D. Bushman, T.Z. DeSantis, G.L. Andersen, R. Knight, PyNAST: a flexible tool for aligning sequences to a template alignment., Bioinformatics. 26 (2010) 266–7. doi:10.1093/bioinformatics/btp636.

[263] J. Oksanen, F.G. Blanchet, R. Kindt, P. Legendre, P.R. Minchin, R.B. O'Hara, G.L. Simpson, P. Solymos, M.H.H. Stevens, H. Wagner, Vegan: Community Ecology Package. R package version 2.3-2, (2015). http://cran.r-project.org/package=vegan.

[264] P.J. McMurdie, S. Holmes, phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data., PLoS One. 8 (2013) e61217. doi:10.1371/journal.pone.0061217.

[265] D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting Linear Mixed-Effects Models using lme4, J. Stat. Softw. 67 (2014) 51.

[266] Y. Benjamini, Y. Hochberg, Controlling the False Discovery Rate: A Practical and Powerful

Approach to Multiple Testing, J. R. Stat. Soc. Ser. B. 57 (1995). doi:10.2307/2346101.

[267] C.A. De Tender, L.I. Devriese, A. Haegeman, S. Maes, T. Ruttink, P. Dawyndt, Bacterial community profiling of plastic litter in the Belgian part of the North Sea, Environ. Sci. Technol. 49 (2015) 9629–9638. doi:10.1021/acs.est.5b01093.

[268] A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, F.O. Glöckner, Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, Nucleic Acids Res. 41 (2013) 1–11. doi:10.1093/nar/gks808.

[269] L. Ovreas, L. Forney, F. Daae, V. Torsvik, Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA, Appl. Envir. Microbiol. 63 (1997) 3367–3373.

[270] S.E. Hook, K.S. Northwood, A.-D.G. Wright, B.W. McBride, Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow., Appl. Environ. Microbiol. 75 (2009) 374–80. doi:10.1128/AEM.01672-08.

[271] Y. Yu, C. Lee, J. Kim, S. Hwang, Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction., Biotechnol. Bioeng. 89 (2005) 670–9. doi:10.1002/bit.20347.

[272] J. Jeyanathan, M. Kirs, R.S. Ronimus, S.O. Hoskin, P.H. Janssen, Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets., FEMS Microbiol. Ecol. 76 (2011) 311–26. doi:10.1111/j.1574-6941.2011.01056.x.

[273] S.E. Denman, N.W. Tomkins, C.S. McSweeney, Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane., FEMS Microbiol. Ecol. 62 (2007) 313–22. doi:10.1111/j.1574-6941.2007.00394.x.

[274] M.W. Friedrich, Methyl-coenzyme M reductase genes: unique functional markers for methanogenic and anaerobic methane-oxidizing Archaea., Methods Enzymol. 397 (2005) 428–42. doi:10.1016/S0076-6879(05)97026-2.

[275] Y. Kong, R. Teather, R. Forster, Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages, FEMS Microbiol. Ecol. 74 (2010) 612–622. doi:10.1111/j.1574-6941.2010.00977.x.

[276] C. Pei, S.-Y. Mao, Y.-F. Cheng, W.-Y. Zhu, Diversity, abundance and novel 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium fractions of Jinnan cattle, Animal. 4 (2010) 20. doi:10.1017/S1751731109990681.

[277] R.J. Gruninger, C.W. Sensen, T.A. McAllister, R.J. Forster, M. Morrison, Diversity of Rumen Bacteria in Canadian Cervids, PLoS One. 9 (2014) e89682. doi:10.1371/journal.pone.0089682.

[278] N.R. Parmar, J. V Solanki, A.B. Patel, T.M. Shah, A.K. Patel, S. Parnerkar, J.I.N. Kumar, C.G. Joshi, Metagenome of Mehsani buffalo rumen microbiota: an assessment of variation in feed-dependent phylogenetic and functional classification., J. Mol. Microbiol. Biotechnol. 24 (2014) 249–61. doi:10.1159/000365054.

[279] E. Ransom-Jones, D.L. Jones, A.J. McCarthy, J.E. McDonald, The Fibrobacteres: an Important Phylum of Cellulose-Degrading Bacteria, Microb. Ecol. 63 (2012) 267–281. doi:10.1007/s00248-011-9998-1.

[280] W.H. Schwarz, The cellulosome and cellulose degradation by anaerobic bacteria, Appl. Microbiol. Biotechnol. 56 (2001) 634–649. doi:10.1007/s002530100710.

[281] M.L. Palop, S. Valles, F. Pinaga, A. Flors, Isolation and Characterization of an Anaerobic, Cellulolytic Bacterium, Clostridium celerecrescens sp. nov., Int. J. Syst. Bacteriol. 39 (1989) 68–71. doi:10.1099/00207713-39-1-68.

[282] B.S. Shane, L. Gouws, A. Kistner, Cellulolytic bacteria occurring in the rumen of sheep conditioned to low-protein teff hay., J. Gen. Microbiol. 55 (1969) 445–57. doi:10.1099/00221287-55-3-445.

[283] D.J. Grilli, M.E. Cerón, S. Paez, V. Egea, L. Schnittger, S. Cravero, M.S. Escudero, L. Allegretti, G.N. Arenas, Isolation of Pseudobutyrivibrio ruminis and Pseudobutyrivibrio xylanivorans from rumen of Creole goats fed native forage diet., Folia Microbiol. (Praha). 58 (2013) 367–73. doi:10.1007/s12223-012-0219-1.

[284] T. Cepeljnik, M.T. Rincón, H.J. Flint, R. Marinsek-Logar, Xyn11A, a multidomain multicatalytic enzyme from Pseudobutyrivibrio xylanivorans Mz5T., Folia Microbiol. (Praha). 51 (2006) 263–7.

[285] H. Patterson, R. Irvin, J.W. Costerton, K.J. Cheng, Ultrastructure and adhesion properties of Ruminococcus albus., J. Bacteriol. 122 (1975) 278–287. http://jb.asm.org/content/122/1/278.abstract (accessed February 23, 2016).

[286] M. Morrison, Adhesion to cellulose by Ruminococcus albus: a combination of cellulosomes and Pil-proteins?, FEMS Microbiol. Lett. 185 (2000) 109–115. doi:10.1016/S0378-1097(00)00040-9.

[287] D. Debroas, G. Blanchart, Interactions between proteolytic and cellulolytic rumen bacteria during hydrolysis of plant cell wall protein., Reprod. Nutr. Dev. 33 (1993) 283–8. http://www.ncbi.nlm.nih.gov/pubmed/8216756 (accessed February 24, 2016).

[288] K.L. Anderson, Biochemical analysis of starch degradation by Ruminobacter amylophilus 70., Appl. Environ. Microbiol. 61 (1995) 1488–91.

[289] R.B. Hespell, The Genera Succinivibrio and Succinimonas, in: A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.-H. Schleifer (Eds.), The Prokaryotes, Springer New York, New York, NY, 1992: pp. 3979–3982. doi:10.1007/978-1-4757-2191-1_60.

[290] R.M. Soo, C.T. Skennerton, Y. Sekiguchi, M. Imelfort, S.J. Paech, P.G. Dennis, J.A. Steen, D.H. Parks, G.W. Tyson, P. Hugenholtz, An expanded genomic representation of the phylum cyanobacteria., Genome Biol. Evol. 6 (2014) 1031–45. doi:10.1093/gbe/evu073.

[291] B. Linehan, C.C. Scheifinger, M.J. Wolin, Nutritional Requirements of Selenomonas ruminantium for Growth on Lactate, Glycerol, or Glucose., Appl. Environ. Microbiol. 35 (1978) 317–22.

[292] R. Danielsson, A. Schnürer, V. Arthurson, J. Bertilsson, Methanogenic population and CH_4 production in swedish dairy cows fed different levels of forage., Appl. Environ. Microbiol. 78 (2012) 6172–9. doi:10.1128/AEM.00675-12.

[293] S.C. Fernando, H.T. Purvis, F.Z. Najar, L.O. Sukharnikov, C.R. Krehbiel, T.G. Nagaraja, B.A. Roe, U. Desilva, Rumen microbial population dynamics during adaptation to a high-grain diet., Appl. Environ. Microbiol. 76 (2010) 7482–90. doi:10.1128/AEM.00388-10.

[294] M.J. Ellison, G.C. Conant, R.R. Cockrum, K.J. Austin, H. Truong, M. Becchi, W.R. Lamberson, K.M. Cammack, Diet alters both the structure and taxonomy of the ovine gut microbial ecosystem., DNA Res. 21 (2014) 115–25. doi:10.1093/dnares/dst044.

[295] B.A. Dehority, J.A. Grubb, Effect of short-term chilling of rumen contents on viable bacterial numbers., Appl. Environ. Microbiol. 39 (1980) 376–81.

[296] S.. Martin-Orue, J. Balcells, F. Zakraoui, C. Castrillo, Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: effect of detachment procedure, Anim. Feed Sci. Technol. 71 (1998) 269–282. doi:10.1016/S0377-8401(97)00156-9.

[297] A.R. Cook, P.W. Riley, H. Murdoch, P.N. Evans, I.R. McDonald, Howardella ureilytica gen. nov., sp. nov., a Gram-positive, coccoid-shaped bacterium from a sheep rumen., Int. J. Syst. Evol. Microbiol. 57 (2007) 2940–5. doi:10.1099/ijs.0.64819-0.

[298] G. Henderson, F. Cox, S. Kittelmann, V.H. Miri, M. Zethof, S.J. Noel, G.C. Waghorn, P.H. Janssen, Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities., PLoS One. 8 (2013) e74787. doi:10.1371/journal.pone.0074787.

[299] NIR, Belgium's Greenhouse Gas Inventory (1990-2015), (2017). http://www.klimaat.be/files/8014/9312/4006/NIR_2017_Belgium_07042017.pdf (accessed August 21, 2017).

[300] FOD Economie, Kerncijfers landbouw: Veestapel runderenen België, (2015). http://melkveebedrijf.be/wp-content/uploads/sites/3/2016/03/TELLING-RUNDEREN-NOV-2015.pdf (accessed February 14, 2017).

[301] V. Campens, D. Van Gijseghem, T. Van Bogaert, Sectoranalyse van de vleeshouderij in Vlaanderen, (2008). http://lv.vlaanderen.be/sites/default/files/attachments/rundveesector.pdf (accessed February 14, 2017).

[302] L.O. Fiems, Double Muscling in Cattle: Genes, Husbandry, Carcasses and Meat., Anim. an Open Access J. from MDPI. 2 (2012) 472–506. doi:10.3390/ani2030472.

[303] S. De Campeneere, L.. Fiems, C.. Boucqué, Energy and protein requirements of Belgian Blue double-muscled bulls, Anim. Feed Sci. Technol. 90 (2001) 153–167. doi:10.1016/S0377-8401(01)00217-6.

[304] R.S. Hegarty, J.P. Goopy, R.M. Herd, B. McCorkell, Cattle selected for lower residual feed intake have reduced daily methane production, J. Anim. Sci. 85 (2007) 1479. doi:10.2527/jas.2006-236.

[305] C. Fitzsimons, D.A. Kenny, M.H. Deighton, A.G. Fahey, M. McGee, Methane emissions, body composition, and rumen fermentation traits of beef heifers differing in residual feed intake, J. Anim. Sci. 91 (2013) 5789–5800. doi:10.2527/jas.2013-6956.

[306] R.W. Todd, M.B. Altman, N.A. Cole, H.M. Waldrip, Methane Emissions from a Beef Cattle Feedyard during Winter and Summer on the Southern High Plains of Texas, J. Environ. Qual. 43 (2014) 1125. doi:10.2134/jeq2013.09.0386.

[307] M.E.Z. Mercadante, A.P. de M. Caliman, R.C. Canesin, S.F.M. Bonilha, A. Berndt, R.T.S. Frighetto, E. Magnani, R.H. Branco, M.E.Z. Mercadante, A.P. de M. Caliman, R.C. Canesin, S.F.M. Bonilha, A. Berndt, R.T.S. Frighetto, E. Magnani, R.H. Branco, Relationship between residual feed intake and enteric methane emission in Nellore cattle, Rev. Bras. Zootec. 44 (2015) 255–262. doi:10.1590/S1806-92902015000700004.

[308] J. Castro-Montoya, N. Peiren, J.W. Cone, B. Zweifel, V. Fievez, S. De Campeneere, In vivo and in vitro effects of a blend of essential oils on rumen methane mitigation, Livest. Sci. 180 (2015) 134–142. doi:10.1016/j.livsci.2015.08.010.

[309] M. Mathot, V. Decruyenaere, D. Stilmant, R. Lambert, Effect of cattle diet and manure storage conditions on carbon dioxide, methane and nitrous oxide emissions from tie-stall barns and stored solid manure, Agric. Ecosyst. Environ. 148 (2012) 134–144. doi:10.1016/j.agee.2011.11.012.

[310] A.N. Hristov, J. Oh, F. Giallongo, T. Frederick, H. Weeks, P.R. Zimmerman, M.T. Harper, R.A. Hristova, R.S. Zimmerman, A.F. Branco, The Use of an Automated System (GreenFeed) to Monitor Enteric Methane and Carbon Dioxide Emissions from Ruminant Animals., J. Vis. Exp. (2015). doi:10.3791/52904.

[311] S. Tamminga, W.M. Van Straalen, A.P.J. Subnel, R.G.M. Meijer, A. Steg, C.J.G. Wever, M.C. Blok, The Dutch protein evaluation system: the DVE/OEB-system, Livest. Prod. Sci. 40 (1994) 139–155. doi:10.1016/0301-6226(94)90043-4.

[312] J.L. De Boever, K. Goossens, N. Peiren, J. Swanckaert, B. Ampe, D. Reheul, D.L. De Brabander, S. De Campeneere, L. Vandaele, The effect of maize silage type on the performances and methane emission of dairy cattle, J. Anim. Physiol. Anim. Nutr. (Berl). 101 (2017) e246–e256. doi:10.1111/jpn.12598.

[313] J.N. Paulson, O.C. Stine, H.C. Bravo, M. Pop, Differential abundance analysis for microbial marker-gene surveys, Nat. Methods. 10 (2013) 1200–1202. doi:10.1038/nmeth.2658.

[314] Y. Chen, D. McCarthy, M. Ritchie, M. Robinson, K.G. Smyth, EdgeR: differential expression analysis of digital gene expression data, (2008). http://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf (accessed January 31, 2017).

[315] K. Li, M. Bihan, B.A. Methé, Analyses of the stability and core taxonomic memberships of the human microbiome., PLoS One. 8 (2013) e63139. doi:10.1371/journal.pone.0063139.

[316] J. Madsen, B. Bjerg, T. Hvelplund, M. Weisbjerg, P. Lund, Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants, 2010. doi:10.1016/j.livsci.2010.01.001.

[317] L.O. Fiems, B.G. Cottyn, C. V. Boucqué, D.F. Bogaerts, C. Eenaeme, J.M. Vanacker, Effect of beef type, body weight and dietary protein content on voluntary feed intake, digestibility, blood and urine metabolites and nitrogen retention, J. Anim. Physiol. Anim. Nutr. (Berl). 77 (1997) 1–9. doi:10.1111/j.1439-0396.1997.tb00731.x.

[318] L.O. Fiems, J.L. De Boever, J.M. Vanacker, Effect of supplementation on the performance of grazing Belgian Blue double-muscled heifers, Animal. 7 (2013) 1806–1815. doi:10.1017/S1751731113001468.

[319] L.. Fiems, D.. De Brabander, Optimum growth rate of Belgian Blue double-muscled replacement heifers, S. Afr. J. Anim. Sci. 39 (2010). doi:10.4314/sajas.v39i1.61179.

[320] J. Madsen, M.F. Bertelsen, Methane production by red-necked wallabies (Macropus rufogriseus), J. Anim. Sci. 90 (2012) 1364–1370. doi:10.2527/jas.2011-4011.

[321] J.A. Rooke, R.J. Wallace, C.-A. Duthie, N. McKain, S.M. de Souza, J.J. Hyslop, D.W. Ross, T. Waterhouse, R. Roehe, Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype., Br. J. Nutr. 112 (2014) 398–407. doi:10.1017/S0007114514000932.

[322] L.M. Cersosimo, M.L. Bainbridge, J. Kraft, A.-D.G. Wright, Influence of periparturient and postpartum diets on rumen methanogen communities in three breeds of primiparous dairy cows, BMC Microbiol. 16 (2016) 78. doi:10.1186/s12866-016-0694-7.

[323] I. Tapio, T.J. Snelling, F. Strozzi, R.J. Wallace, The ruminal microbiome associated with methane emissions from ruminant livestock, J. Anim. Sci. Biotechnol. 8 (2017) 7. doi:10.1186/s40104-017-0141-0.

[324] F. Rubino, C. Carberry, S. M Waters, D. Kenny, M.S. McCabe, C.J. Creevey, Divergent

functional isoforms drive niche specialisation for nutrient acquisition and use in rumen microbiome, ISME J. 11 (2017) 932–944. doi:10.1038/ismej.2016.172.

[325] D. Griffin, M.M. Chengappa, J. Kuszak, D.S. McVey, Bacterial Pathogens of the Bovine Respiratory Disease Complex, Vet. Clin. North Am. Food Anim. Pract. 26 (2010) 381–394. doi:10.1016/j.cvfa.2010.04.004.

[326] M. Mahu, B. Valgaeren, B. Pardon, P. Deprez, F. Haesebrouck, F. Boyen, Non-haemolytic Mannheimia haemolytica as a cause of pleuropneumonia and septicemia in a calf, Vet. Microbiol. 180 (2015) 157–160. doi:10.1016/j.vetmic.2015.08.019.

[327] N.G. Regalado, G. Martin, S.J. Antony, Acinetobacter lwoffii: Bacteremia associated with acute gastroenteritis, Travel Med. Infect. Dis. 7 (2009) 316–317. doi:10.1016/j.tmaid.2009.06.001.

[328] A. Bassett, Animal Welfare Approved. Technical Advice Fact sheet No 1. Welfare and Belgian Blue Cattle, (2009).

[329] J. Neu, J. Rushing, Cesarean Versus Vaginal Delivery: Long-term Infant Outcomes and the Hygiene Hypothesis, Clin. Perinatol. 38 (2011) 321–331. doi:10.1016/j.clp.2011.03.008.

[330] R. Nagpal, H. Tsuji, T. Takahashi, K. Nomoto, K. Kawashima, S. Nagata, Y. Yamashiro, Ontogenesis of the Gut Microbiota Composition in Healthy, Full-Term, Vaginally Born and Breast-Fed Infants over the First 3 Years of Life: A Quantitative Bird's-Eye View., Front. Microbiol. 8 (2017) 1388. doi:10.3389/fmicb.2017.01388.

[331] A. Moya-Pérez, P. Luczynski, I.B. Renes, S. Wang, Y. Borre, C. Anthony Ryan, J. Knol, C. Stanton, T.G. Dinan, J.F. Cryan, Intervention strategies for cesarean section-induced alterations in the microbiota-gut-brain axis., Nutr. Rev. 75 (2017) 225–240. doi:10.1093/nutrit/nuw069.

[332] K. Tajima, R.I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, Y. Benno, Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR., Appl. Environ. Microbiol. 67 (2001) 2766–74. doi:10.1128/AEM.67.6.2766-2774.2001.

[333] A. Belanche, M. Doreau, J.E. Edwards, J.M. Moorby, E. Pinloche, C.J. Newbold, Shifts in the Rumen Microbiota Due to the Type of Carbohydrate and Level of Protein Ingested by Dairy Cattle Are Associated with Changes in Rumen Fermentation, J. Nutr. 142 (2012) 1684–1692. doi:10.3945/jn.112.159574.

[334] O. AlZahal, F. Li, L. Luo Guan, N.D. Walker, B.W. McBride, Factors influencing ruminal bacterial community diversity and composition and microbial fibrolytic enzyme abundance in lactating dairy cows with a focus on the role of active dry yeast, J. Dairy Sci. 100 (2017). doi:10.3168/jds.2016-11473.

[335] G. Getachew, H.P.. Makkar, K. Becker, Method of polyethylene glycol application to tannin-containing browses to improve microbial fermentation and efficiency of microbial protein synthesis from tannin-containing browses, Anim. Feed Sci. Technol. 92 (2001) 51–57. doi:10.1016/S0377-8401(01)00250-4.

[336] J. Voigt, H. Steger, Zur quantitativen Bestimmung von Ammoniak, Harnstoff und Ketonkörperns in biologischem Material mit Hilfe eines modifizierten Mikrodiffusionsgefässes, Arch. Tierernahr. 17 (1967) 289–293.

[337] H. Morita, C. Shiratori, M. Murakami, H. Takami, H. Toh, Y. Kato, F. Nakajima, M. Takagi, H. Akita, T. Masaoka, M. Hattori, Sharpea azabuensis gen. nov., sp. nov., a Gram-positive, strictly anaerobic bacterium isolated from the faeces of thoroughbred horses, Int. J. Syst. Evol. Microbiol. 58 (2008) 2682–2686. doi:10.1099/ijs.0.65543-0.

[338] V.H. Varel, H.J. Jung, Influence of forage phenolics on ruminal fibrolytic bacteria and in vitro

fiber degradation., Appl. Environ. Microbiol. 52 (1986) 275–80.

[339] T. Nagaraja, M. Taylor, Susceptibility and Resistance of Ruminal Bacteria to Antimicrobial Feed Additives, Appl. Abd Environ. Microbiol. 53 (1987) 1620–1625.

[340] C. Winter, T. Bouvier, M.G. Weinbauer, T.F. Thingstad, Trade-offs between competition and defense specialists among unicellular planktonic organisms: the "killing the winner" hypothesis revisited., Microbiol. Mol. Biol. Rev. 74 (2010) 42–57. doi:10.1128/MMBR.00034-09.

[341] B. Koskella, S. Meaden, Understanding Bacteriophage Specificity in Natural Microbial Communities, Viruses. 5 (2013) 806–823. doi:10.3390/v5030806.

[342] I. Luque, C. Tarradas, L. Carrasco, E. Torroella, C. Artigas, A. Perea, Effectiveness of doxycycline in the prevention of an experimental infection with Actinobacillus pleuropneumoniae in pigs., J. Vet. Med. B. Infect. Dis. Vet. Public Health. 47 (2000) 445–51.

[343] E. Bousquet, P. Pommier, S. Wessel-Robert, H. Morvan, H. Benoit-Valiergue, A. Laval, Efficacy of doxycycline in feed for the control of pneumonia caused by Pasteurella multocida and Mycoplasma hyopneumoniae in fattening pigs., Vet. Rec. 143 (1998) 269–72.

[344] WHO, Critically important antimicrobials for human medicine, WHO. (2017).

[345] J. Desloover, J. De Vrieze, M. Van de Vijver, J. Mortelmans, R. Rozendal, K. Rabaey, Electrochemical nutrient recovery enables ammonia toxicity control and biogas desulfurization in anaerobic digestion., Environ. Sci. Technol. 49 (2015) 948–55. doi:10.1021/es504811a.

[346] C.W. Knapp, J. Dolfing, D.W. Graham, Evidence of Increasing Antibiotic Resistance Gene Abundances in Archived Soils since 1940, Environ. Sci. Technol. 44 (2010) 580–587. doi:10.1021/es901221x.

[347] M. Guarddon, J.M. Miranda, J.A. Rodríguez, B.I. Vázquez, A. Cepeda, C.M. Franco, Real-time polymerase chain reaction for the quantitative detection of tetA and tetB bacterial tetracycline resistance genes in food, Int. J. Food Microbiol. 146 (2011) 284–289. doi:10.1016/j.ijfoodmicro.2011.02.026.

[348] N. Peak, C.W. Knapp, R.K. Yang, M.M. Hanfelt, M.S. Smith, D.S. Aga, D.W. Graham, Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies., Environ. Microbiol. 9 (2007) 143–51. doi:10.1111/j.1462-2920.2006.01123.x.

[349] S. Koike, I.G. Krapac, H.D. Oliver, A.C. Yannarell, J.C. Chee-Sanford, R.I. Aminov, R.I. Mackie, Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period., Appl. Environ. Microbiol. 73 (2007) 4813–23. doi:10.1128/AEM.00665-07.

[350] M. Munir, I. Xagoraraki, Levels of antibiotic resistance genes in manure, biosolids, and fertilized soil., J. Environ. Qual. 40 (2011) 248–255. doi:10.2134/jeq2010.0209.

[351] G.V. Schmidt, A. Mellerup, L.E. Christiansen, M. Ståhl, J.E. Olsen, Ø. Angen, Sampling and Pooling Methods for Capturing Herd Level Antibiotic Resistance in Swine Feces using qPCR and CFU Approaches., PLoS One. 10 (2015) e0131672. doi:10.1371/journal.pone.0131672.

[352] S. Pakpour, S. Jabaji, M.R. Chénier, Frequency of Antibiotic Resistance in a Swine Facility 2.5 Years After a Ban on Antibiotics, Microb. Ecol. 63 (2012) 41–50. doi:10.1007/s00248-011-9954-0.

[353] A. van Hoek, A. Margolles, K. Domig, J. Korhonen, J. Zycka-Krzesinska, J. Bardowski, M.

Danielsen, G. Huys, L. Morelli, H. Aarts, Molecular assessment of erythromycin and tetracycline resistance genes in lactic acid bacteria and bifidobacteria and their relation to the phenotypic resistance, Int. J. PROBIOTICS PREBIOTICS. 3 (2008) 271–280.

[354] M. Dec, R. Urban-Chmiel, D. Stępień-Pyśniak, A. Wernicki, Assessment of antibiotic susceptibility in Lactobacillus isolates from chickens., Gut Pathog. 9 (2017) 54. doi:10.1186/s13099-017-0203-z.

[355] K. Cauwerts, F. Pasmans, L.A. Devriese, F. Haesebrouck, A. Decostere, Cloacal Lactobacillus isolates from broilers often display resistance toward tetracycline antibiotics., Microb. Drug Resist. 12 (2006) 284–288. doi:10.1089/mdr.2006.12.284.

[356] N.T. Hoa, T.T. Chieu, H.D. Nghia, N.T. Mai, P.H. Anh, M. Wolbers, S. Baker, J.I. Campbell, N.V. Chau, T.T. Hien, J. Farrar, C. Schultsz, The antimicrobial resistance patterns and associated determinants in Streptococcus suisisolated from humans in southern Vietnam, 1997-2008, BMC Infect. Dis. 11 (2011) 6. doi:10.1186/1471-2334-11-6.

[357] R. Nomoto, L.H.T. Tien, T. Sekizaki, R. Osawa, Antimicrobial susceptibility of Streptococcus gallolyticus isolated from humans and animals., Jpn. J. Infect. Dis. 66 (2013) 334–6. http://www.ncbi.nlm.nih.gov/pubmed/23883848 (accessed October 31, 2017).

[358] T.B. Stanton, J.S. McDowall, M.A. Rasmussen, Diverse tetracycline resistance genotypes of Megasphaera elsdenii strains selectively cultured from swine feces., Appl. Environ. Microbiol. 70 (2004) 3754–7. doi:10.1128/AEM.70.6.3754-3757.2004.

[359] C.M. Melville, R. Brunel, H.J. Flint, K.P. Scott, The Butyrivibrio fibrisolvens tet(W) gene is carried on the novel conjugative transposon TnB1230, which contains duplicated nitroreductase coding sequences., J. Bacteriol. 186 (2004) 3656–9. doi:10.1128/JB.186.11.3656-3659.2004.

[360] T.M. Barbosa, K.P. Scott, H.J. Flint, Evidence for recent intergeneric transfer of a new tetracycline resistance gene, tet(W), isolated from Butyrivibrio fibrisolvens, and the occurrence of tet(O) in ruminal bacteria., Environ. Microbiol. 1 (1999) 53–64. http://www.ncbi.nlm.nih.gov/pubmed/11207718 (accessed October 31, 2017).

[361] A. Villedieu, M.L. Diaz-Torres, N. Hunt, R. Mcnab, D.A. Spratt, M. Wilson, P. Mullany, Prevalence of Tetracycline Resistance Genes in Oral Bacteria, Antimicrob. Agents Chemother. 47 (2003) 878–882. doi:10.1128/AAC.47.3.878–882.2003.

[362] M.E.H. Bos, F.J. Taverne, I.M. van Geijlswijk, J.W. Mouton, D.J. Mevius, D.J.J. Heederik, Consumption of Antimicrobials in Pigs, Veal Calves, and Broilers in The Netherlands: Quantitative Results of Nationwide Collection of Data in 2011, PLoS One. 8 (2013) e77525. doi:10.1371/journal.pone.0077525.

[363] M.E. Filippitzi, B. Callens, B. Pardon, D. Persoons, J. Dewulf, Antimicrobial use in pigs, broilers and veal calves in Belgium, Vlaams Diergeneeskd. Tijdschr. 83 (2014) 215–224.

[364] EC, Council Directive 90/167/EEC of 26 March 1990 laying down the conditions governing the preparation, placing on the market and use of medicated feedingstuffs in the Community, (1990). http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A31990L0167 (accessed August 1, 2016).

[365] EC, Proposal for a REGULATION OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on the manufacture, placing on the market and use of medicated feed and repealing Council Directive 90/167/EEC, (2014). http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:52014PC0556&from=EN (accessed August 1, 2016).

[366] J. Dewulf, B. Hoet, D. Minne, Belgian Veterinary Surveillance of Antibacterial Consumption. National report 2015, (2016).

http://www.belvetsac.ugent.be/pages/home/BelvetSAC_report_2015.pdf (accessed July 6, 2017).

[367] ESVAC, Fifth ESVAC report: Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2013., EMA/38934/2015. (2015). http://www.amcra.be/sites/default/files/bestanden/Fifth ESVAC report.pdf (accessed July 6, 2017).

[368] S. Borràs, R. Companyó, M. Granados, J. Guiteras, A.M. Pérez-Vendrell, J. Brufau, M. Medina, J. Bosch, Analysis of antimicrobial agents in animal feed, TrAC Trends Anal. Chem. 30 (2011) 1042–1064. doi:10.1016/j.trac.2011.02.012.

[369] A. Widyasari-Mehta, H.R.K.A. Suwito, R. Kreuzig, Laboratory testing on the removal of the veterinary antibiotic doxycycline during long-term liquid pig manure and digestate storage, Chemosphere. 149 (2016) 154–160. doi:10.1016/j.chemosphere.2016.01.094.

[370] Y. Chen, H. Zhang, Y. Luo, J. Song, Occurrence and assessment of veterinary antibiotics in swine manures: A case study in East China, Chinese Sci. Bull. 57 (2012) 606–614. doi:10.1007/s11434-011-4830-3.

[371] M. Carballo, S. Aguayo, M. González, F. Esperon, A. De La Torre, Environmental Assessment of Tetracycline's Residues Detected in Pig Slurry and Poultry Manure, J. Environ. Prot. (Irvine,. Calif). 7 (2016) 82–92. doi:10.4236/jep.2016.71008.

[372] W. Messens, J. Goris, N. Dierick, L. Herman, M. Heyndrickx, Inhibition of Salmonella typhimurium by medium-chain fatty acids in an in vitro simulation of the porcine cecum., Vet. Microbiol. 141 (2010) 73–80. doi:10.1016/j.vetmic.2009.08.002.

[373] A. Smet, G. Rasschaert, A. Martel, D. Persoons, J. Dewulf, P. Butaye, B. Catry, F. Haesebrouck, L. Herman, M. Heyndrickx, In situ ESBL conjugation from avian to human Escherichia coli during cefotaxime administration, J. Appl. Microbiol. 110 (2011) 541–549. doi:10.1111/j.1365-2672.2010.04907.x.

[374] EUCAST, EUCAST: MIC distributions and ECOFFs, (2016). http://www.eucast.org/mic_distributions_and_ecoffs/ (accessed July 25, 2016).

[375] S.J. Andersen, T. Hennebel, S. Gildemyn, M. Coma, J. Desloover, J. Berton, J. Tsukamoto, C. Stevens, K. Rabaey, Electrolytic Membrane Extraction Enables Production of Fine Chemicals from Biorefinery Sidestreams, Environ. Sci. Technol. 48 (2014) 7135–7142. doi:10.1021/es500483w.

[376] UniProt, UniProtKB, (2016). http://www.uniprot.org/uniprot/ (accessed July 20, 2016).

[377] ARDB, ARDB - Antibiotic Resistance Genes Database, (2009). http://ardb.cbcb.umd.edu (accessed July 20, 2016).

[378] D. Ríos-Covián, P. Ruas-Madiedo, A. Margolles, M. Gueimonde, C.G. de Los Reyes-Gavilán, N. Salazar, Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health., Front. Microbiol. 7 (2016) 185. doi:10.3389/fmicb.2016.00185.

[379] V.H. Varel, S.J. Fryda, I.M. Robinson, Cellulolytic bacteria from pig large intestine., Appl. Environ. Microbiol. 47 (1984) 219–21. http://www.ncbi.nlm.nih.gov/pubmed/6696420 (accessed October 19, 2016).

[380] H.J. Flint, E.A. Bayer, M.T. Rincon, R. Lamed, B.A. White, Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis, Nat. Rev. Microbiol. 6 (2008) 121–131. doi:10.1038/nrmicro1817.

[381] P.S. Stewart, J.W. Costerton, Antibiotic resistance of bacteria in biofilms., Lancet (London,

England). 358 (2001) 135–8.

[382] D.B. Holman, M.R. Chénier, Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine., FEMS Microbiol. Ecol. 85 (2013) 1–13. doi:10.1111/1574-6941.12093.

[383] A.E. van den Bogaard, E.E. Stobberingh, Epidemiology of resistance to antibiotics. Links between animals and humans., Int. J. Antimicrob. Agents. 14 (2000) 327–35.

[384] A.L. Barkovskii, C. Bridges, Erratum to: Persistence and Profiles of Tetracycline Resistance Genes in Swine Farms and Impact of Operational Practices on Their Occurrence in Farms' Vicinities, Water, Air, Soil Pollut. 223 (2012) 3577–3577. doi:10.1007/s11270-012-1168-7.

[385] C.W. Beitel, L. Froenicke, J.M. Lang, I.F. Korf, R.W. Michelmore, J.A. Eisen, A.E. Darling, Strain- and plasmid-level deconvolution of a synthetic metagenome by sequencing proximity ligation products., PeerJ. 2 (2014) e415. doi:10.7717/peerj.415.

[386] S.J. Spencer, M. V Tamminen, S.P. Preheim, M.T. Guo, A.W. Briggs, I.L. Brito, D. A Weitz, L.K. Pitkänen, F. Vigneault, M.Pj. Virta, E.J. Alm, Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers, ISME J. 10 (2016) 427–436. doi:10.1038/ismej.2015.124.

[387] W.M. Dunne, Bacterial adhesion: seen any good biofilms lately?, Clin. Microbiol. Rev. 15 (2002) 155–66. doi:10.1128/cmr.15.2.155-166.2002.

[388] V. Roger, G. Fonty, S. Komisarczuk-Bony, P. Gouet, Effects of Physicochemical Factors on the Adhesion to Cellulose Avicel of the Ruminal Bacteria Ruminococcus flavefaciens and Fibrobacter succinogenes subsp. succinogenes., Appl. Environ. Microbiol. 56 (1990) 3081–7. http://www.ncbi.nlm.nih.gov/pubmed/16348315 (accessed October 20, 2016).

[389] P.S. Stewart, Mechanisms of antibiotic resistance in bacterial biofilms, Int. J. Med. Microbiol. 292 (2002) 107–113. doi:10.1078/1438-4221-00196.

[390] G. Muyzer, K. Smalla, Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology., Antonie Van Leeuwenhoek. 73 (1998) 127–41. http://www.ncbi.nlm.nih.gov/pubmed/9602286 (accessed October 16, 2017).

[391] M.M. Fisher, E.W. Triplett, Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities., Appl. Environ. Microbiol. 65 (1999) 4630–6. http://www.ncbi.nlm.nih.gov/pubmed/10508099 (accessed October 16, 2017).

[392] T.L. Marsh, Culture-Independent Microbial Community Analysis with Terminal Restriction Fragment Length Polymorphism, in: Methods Enzymol., 2005: pp. 308–329. doi:10.1016/S0076-6879(05)97018-3.

[393] P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R.A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G.G. Thallinger, D.J. Van Horn, C.F. Weber, Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities, Appl. Environ. Microbiol. 75 (2009) 7537–7541. doi:10.1128/AEM.01541-09.

[394] C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, F.O. Glockner, The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, Nucleic Acids Res. 41 (2013) D590–D596. doi:10.1093/nar/gks1219.

[395] J.R. Cole, Q. Wang, J.A. Fish, B. Chai, D.M. McGarrell, Y. Sun, C.T. Brown, A. Porras-Alfaro,

C.R. Kuske, J.M. Tiedje, Ribosomal Database Project: data and tools for high throughput rRNA analysis., Nucleic Acids Res. 42 (2014) D633-42. doi:10.1093/nar/gkt1244.

[396] E.C. Giles, J. Kamke, L. Moitinho-Silva, M.W. Taylor, U. Hentschel, T. Ravasi, S. Schmitt, Bacterial community profiles in low microbial abundance sponges, FEMS Microbiol. Ecol. 83 (2013) 232–241. doi:10.1111/j.1574-6941.2012.01467.x.

[397] T. Větrovský, P. Baldrian, The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses., PLoS One. 8 (2013) e57923. doi:10.1371/journal.pone.0057923.

[398] N.-P. Nguyen, T. Warnow, M. Pop, B. White, A perspective on 16S rRNA operational taxonomic unit clustering using sequence similarity, Npj Biofilms Microbiomes. 2 (2016) 16004. doi:10.1038/npjbiofilms.2016.4.

[399] IHMS project, No Title, (n.d.). http://www.microbiome-standards.org/ (accessed September 25, 2017).

[400] EFSA, Use of Whole Genome Sequencing (WGS) of food-borne pathogens for public health protection, (2014). https://publications.europa.eu/en/publication-detail/-/publication/c966c3c6-0aa7-11e6-b713-01aa75ed71a1/language-en (accessed October 19, 2017).

[401] G. Visentin, S. McParland, M. De Marchi, A. McDermott, M.A. Fenelon, M. Penasa, D.P. Berry, Processing characteristics of dairy cow milk are moderately heritable, J. Dairy Sci. 100 (2017) 6343–6355. doi:10.3168/JDS.2017-12642.

[402] C.S. Pinares-Patino, J.C. McEwan, K.G. Dodds, E.A. Cardenas, R.S. Hegarty, J.P. Koolaard, H. Clark, Repeatability of methane emissions from sheep, Anim. Feed Sci. Technol. 166–167 (2011) 210–218. doi:10.1016/J.ANIFEEDSCI.2011.04.068.

[403] S. Giger-Reverdin, D. Sauvant, Study of the between-goat variation in feed efficiency with a high-concentrate diet, Options Méditerranéeenes. (2015). http://prodinra.inra.fr/ft?id=E1441972-215E-4EBA-A9DC-114A8EFDECB5 (accessed September 27, 2017).

[404] W.Z. Yang, K.A. Beauchemin, L.M. Rode, Effects of Grain Processing, Forage to Concentrate Ratio, and Forage Particle Size on Rumen pH and Digestion by Dairy Cows, J. Dairy Sci. 84 (2001) 2203–2216. doi:10.3168/jds.S0022-0302(01)74667-X.

[405] T. Curtis, W. Sloan, Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology, Curr. Opin. Microbiol. 7 (2004) 221–226. doi:10.1016/j.mib.2004.04.010.

[406] M. Rey, F. Enjalbert, S. Combes, L. Cauquil, O. Bouchez, V. Monteils, Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential, J. Appl. Microbiol. 116 (2014) 245–257. doi:10.1111/jam.12405.

[407] S. Langenheder, E.S. Lindström, L.J. Tranvik, Structure and function of bacterial communities emerging from different sources under identical conditions., Appl. Environ. Microbiol. 72 (2006) 212–20. doi:10.1128/AEM.72.1.212-220.2006.

[408] R. de Wit, T. Bouvier, “Everything is everywhere, but, the environment selects”; what did Baas Becking and Beijerinck really say?, Environ. Microbiol. 8 (2006) 755–758. doi:10.1111/j.1462-2920.2006.01017.x.

[409] D.G. Welkie, D.M. Stevenson, P.J. Weimer, ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle, Anaerobe. 16 (2010) 94–100. doi:10.1016/j.anaerobe.2009.07.002.

[410] M. Sandri, C. Manfrin, A. Pallavicini, B. Stefanon, Microbial biodiversity of the liquid fraction of rumen content from lactating cows, Animal. 8 (2014) 572–579. doi:10.1017/S1751731114000056.

[411] D.E. Rico, S.H. Preston, J.M. Risser, K.J. Harvatine, Rapid changes in key ruminal microbial populations during the induction of and recovery from diet-induced milk fat depression in dairy cows, Br. J. Nutr. 114 (2015) 358–367. doi:10.1017/S0007114515001865.

[412] J. De Vrieze, L. Regueiro, R. Props, R. Vilchez-Vargas, R. Jáuregui, D.H. Pieper, J.M. Lema, M. Carballa, Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion., Biotechnol. Biofuels. 9 (2016) 244. doi:10.1186/s13068-016-0652-5.

[413] A. Simón-Soro, M. Guillen-Navarro, A. Mira, Metatranscriptomics reveals overall active bacterial composition in caries lesions., J. Oral Microbiol. 6 (2014) 25443. doi:10.3402/JOM.V6.25443.

[414] A. Wright, P. Kennedy, C.J. O'Neill, A.F. Toovey, S. Popovski, S.M. Rea, C.L. Pimm, L. Klein, Reducing methane emissions in sheep by immunization against rumen methanogens, Vaccine. 22 (2004) 3976–3985. doi:10.1016/j.vaccine.2004.03.053.

[415] Y.J. Williams, S. Popovski, S.M. Rea, L.C. Skillman, A.F. Toovey, K.S. Northwood, A.-D.G. Wright, A vaccine against rumen methanogens can alter the composition of archaeal populations., Appl. Environ. Microbiol. 75 (2009) 1860–6. doi:10.1128/AEM.02453-08.

[416] L. Zhang, X. Huang, B. Xue, Q. Peng, Z. Wang, T. Yan, L. Wang, Immunization against Rumen Methanogenesis by Vaccination with a New Recombinant Protein., PLoS One. 10 (2015) e0140086. doi:10.1371/journal.pone.0140086.

[417] A. Romero-Perez, E.K. Okine, S.M. McGinn, L.L. Guan, M. Oba, S.M. Duval, M. Kindermann, K.A. Beauchemin, Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet, J. Anim. Sci. 93 (2015) 1780. doi:10.2527/jas.2014-8726.

[418] J. Haisan, Y. Sun, L.L. Guan, K.A. Beauchemin, A. Iwaasa, S. Duval, D.R. Barreda, M. Oba, The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation, J. Dairy Sci. 97 (2014) 3110–3119. doi:10.3168/jds.2013-7834.

[419] M. Demerec, Pattere-ns of bacterial resistance to penicillin, aureomycin, and streptomycin, J. Clin. Invest. 28 (1949) 891–3. doi:10.1172/JCI102173.

[420] N. Händel, J.M. Schuurmans, S. Brul, B.H. ter Kuile, Compensation of the metabolic costs of antibiotic resistance by physiological adaptation in Escherichia coli., Antimicrob. Agents Chemother. 57 (2013) 3752–62. doi:10.1128/AAC.02096-12.

[421] R. Props, F.-M. Kerckhof, P. Rubbens, J. De Vrieze, E. Hernandez Sanabria, W. Waegeman, P. Monsieurs, F. Hammes, N. Boon, Absolute quantification of microbial taxon abundances, ISME J. 11 (2017) 584–587. doi:10.1038/ismej.2016.117.

[422] J.R. Stokell, T.J. Hamp, T.R. Steck, Examining changes in bacterial abundance in complex communities using next-generation sequencing is enhanced with quantitative PCR, Antonie Van Leeuwenhoek. 109 (2016) 1161–1166. doi:10.1007/s10482-016-0707-4.

[423] T.W. V Daniels, G.B. Rogers, F.A. Stressmann, C.J. Van Der Gast, K.D. Bruce, G.R. Jones, G.J. Connett, J.P. Legg, M.P. Carroll, Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis, J. Cyst. Fibros. 12 (2013) 22–28. doi:10.1016/j.jcf.2012.05.008.

[424] F. Stämmler, J. Gläsner, A. Hiergeist, E. Holler, D. Weber, P.J. Oefner, A. Gessner, R. Spang,

Adjusting microbiome profiles for differences in microbial load by spike-in bacteria., Microbiome. 4 (2016) 28. doi:10.1186/s40168-016-0175-0.

[425] T. Zuidema, F.L. van Holthoon, H.J. van Egmond, J. Hooglugt, P. Bikker, H. Aarts, E. Olde, Omvang en implicaties van antibiotica-versleping in mengvoeders voor varkens, (2010) 1–71. http://edepot.wur.nl/157718 (accessed April 3, 2017).

[426] F. Putier, Evaluation of cross-contamination in livestock feed delivery trucks, Tech. Kraftfutter / Feed Mag. 9-10/2010. (2010).

[427] L.E.J. Peeters, S. Croubels, G. Rasschaert, H. Imberechts, E. Daeseleire, J. Dewulf, M. Heyndrickx, P. Butaye, F. Haesebrouck, A. Smet, Effect of residual doxycycline concentrations on resistance selection and transfer in porcine commensal escherichia coli, Int. J. Antimicrob. Agents. (2017). doi:10.1016/j.ijantimicag.2017.04.018.

[428] N. Rose, M. Andraud, The use of vaccines to control pathogen spread in pig populations, Porc. Heal. Manag. 3 (2017) 8. doi:10.1186/s40813-017-0053-6.

[429] C. Rojo-Gimeno, M. Postma, J. Dewulf, H. Hogeveen, L. Lauwers, E. Wauters, Farm-economic analysis of reducing antimicrobial use whilst adopting improved management strategies on farrow-to-finish pig farms, Prev. Vet. Med. 129 (2016). doi:10.1016/j.prevetmed.2016.05.001.

Summary
Samenvatting

SUMMARY

The demand for animal-derived products has more than tripled over the past fifty years due to the exponential growth of the human population and the increased prosperity in many countries. As a result, livestock production co-evolved towards more intensive production systems to meet the rising demands. This intensification is reflected in the dense and indoor housing of animals, the transition to energy and protein-rich diets to boost growth and/or production and the frequent use of antibiotics to prevent or treat infections. A side-effect of intensive cattle farming and the increased number of animals is the massive emissions of enteric methane, a potent greenhouse gas, and the frequent antibiotic use in pig husbandry causes an enrichment of resistant bacteria that can spread to the environment, farmers and consumers. Both of these topical problems find their origin in the intestinal microbial ecosystems of these animals.

This PhD dissertation consists of two parts: (1.) Identifying the factors that shape the rumen bacterial and methanogen communities and their metabolic activities, with the link to methane productions, and (2.) investigating the influences of cross-contamination of feed with doxycycline on the intestinal microbial communities of pigs. The overarching goal was to optimize and implement metagenomics techniques to identify taxonomic groups in these microbial communities, mapping shifts in the community compositions as response to external and internal factors and linking the observed taxonomic changes to functionalities.

A good understanding of the rumen ecosystem is essential to increase productivity or decrease methane production. Rumen sample collection is key in these studies, yet complicated by the complexity of the rumen ecosystem, which comprises different environments: the solid matter, the rumen fluid and the epithelium. The aim of **Chapter 2** was to optimize protocols for specific sampling of the above mentioned rumen environments, to identify environment-specific species and to evaluate sample types for their ability to represent the whole rumen ecosystem. Five sample types were collected from four cannulated cows: crude solids (S), the eluted solid-adherent fraction (Ad), free-living species in the crude rumen liquid (CRL), strained liquid samples (Lq) and epimural scrapings (Ep). The results indicated that the liquid and solid-adherent environments were distinguished mainly by the differential abundance of specific taxonomic groups. Cellulolytic bacteria that pioneer biofilm formation and secondary colonizers were prevalent in solid-adherent samples, while species in the fluid samples were

primarily identified as consumers of soluble nutrients. Also methanogen species were found to have a preference for either a solid-adherent or free-living occurrence. The epimural environment was characterized by a very distinct microbial profile. Ten bacterial families and two methanogen genera were almost exclusively found in this environment. The CRL sample type best represents the whole rumen ecosystem as it includes solid-associated species that disperse from mature biofilms and planktonic species.

In **Chapter 3**, the hypothesis that breed-related characteristics could select for a breed-specific microbiome is investigated. The microbial community composition and methane emissions of the Holstein-Friesian (HF) dairy breed and the Double-muscled Belgian Blue (DMBB) beef breed were compared under conditions of equal diet composition, environment and physiological stage. The absolute methane production, expressed a gram CH_4 per day, was significantly higher for HF heifers compared to DMBB heifers, however this difference did not remain when expressing methane emissions per kg dry matter intake. Although both breeds accommodated a common core of taxonomic groups, the bacterial communities showed a breed specific composition due to differential abundance of specific species belonging to the main taxonomic groups and the presence of a few species of minor taxonomic groups that were significantly associated with one of both breeds. In contrast to the bacterial communities, the methanogen community was consistent and stable between breeds and at different sampling times. Our results suggest that breed-related factors influenced the bacterial community composition, while the variation in methane emission levels could be attributed mainly to the feed intake of the animals.

While Chapter 3 focused on the influence of breed on the rumen microbiome, **Chapter 4** aimed at identifying the host's influence on the rumen methanogen and bacterial communities. The extent of the host's influence was examined by following the composition of the microbial communities of four cows after a ruminal content transfer, under conditions of equal nutrition and physiological stage. Out of the four cannulated HF cows (mid-lactation), one donor cow was selected based on its slightly higher methane production. The rumen content of the donor was thoroughly removed and used as inoculum for the emptied rumen of the donor itself and three acceptor cows. The response to this perturbation of the rumen ecosystem was different between cows. The donor and one of the acceptor cows had a brief depression in feed intake, resulting in lower methane emissions and altered volatile fatty acid (VFA) proportions. These short-term changes were further reflected in the bacterial community during the first two days after transfer: the richness significantly decreased and novel taxa gained the opportunity to

dominate the community. Following these circumstances, the rumen bacterial community underwent several autogenic successions in its search for a new steady state. Further, the fermentation metrics of the two other acceptor cows were not affected. Their rumen bacterial composition initially maintained the composition of the donor, but over time the bacterial community reached a new dynamic equilibrium that resembled neither the donor nor the original composition. These results suggests that the rumen bacterial community can restore quickly after a severe perturbation. In the absence of dietary influence, the composition was not solely host specific, instead the bacterial community was partly influenced by host related factors but dynamic over time resulting in a well-balanced ecosystem with a core of stable and omnipresent species and transiently successive species. Opposite to the bacteria, the methanogenic communities were unaffected by host effects and were stable over time.

Chapters 2 to 4 described experiments where metabarcoding was used to characterised the rumen microbiome and identify the factors that shape its community compositions. In the second part of this PhD thesis, the effect of cross-contamination of doxycycline in pig feed was investigated on the intestinal and fecal microbial communities, focussing on the impact on the microbial community composition, metabolic activity and the number of resistant bacteria and resistance genes.

A preliminary *in vivo* experiment (**Chapter 5**), performed at the CODA, investigated the intestinal and fecal concentrations of antibiotics when pigs are fed a "cross-contaminated" diet, i.e. containing 3% of the maximum recommended those of the specific antibiotic. Doxycycline (DOX) reached a stable concentration of about 4 mg kg^{-1} in the manure of treated pigs after four days of feeding the "contaminated" diet. Concomitantly, tetracycline resistance genes *tet*(W) and *tet*(L) significantly increased, whereas other tested resistance genes *tet*(O), *tet*(Q), *tet*(A), *tet*(M), *tet*(B) were not enriched during treatment. The fecal microbial community composition was unaffected by the continuous influx of subtherapeutic doxycycline and no taxonomic groups were significantly enriched during DOX treatment, as compared to the control group. Only a short-term effect was observed on the microbial richness and diversity, which was at its lowest on the fourth day of administration. The carry-over of 3% of a therapeutic dose of DOX induced the enrichment of only a few tetracycline resistance genes but did not influence the composition of the fecal microbial communities of pigs.

The *in vivo* experiment (Chapter 5) indicated intestinal concentrations in the range of 1 and 4 mg kg^{-1} DOX in the cecum of the treated pigs (published by Peeters et al. (2016)). The

experiments described in **Chapter 6** were designed to investigate the effect of these intestinal concentrations on the intestinal microbial ecosystem. Beside the concentrations found in the intestines as a result of cross-contamination, i.e. 1 and 4 mg l^{-1} feed medium, also a reference concentration of 16 mg l^{-1} was tested. These concentrations were continuously administered to a chemostat, simulating the microbial ecosystem of the pig cecum and inoculated with cecal content of organically grown pigs. The administration of even the lowest DOX concentration caused a significant decrease in bacterial activity, while the microbial community profile seemed unaffected by any of the concentrations. A concentration of 1 mg l^{-1} DOX caused a minor selection pressure for tetracycline resistant *E. coli* but not for other groups enumerated with plate cultivation, while 4 mg l^{-1} induced major enrichment of tetracycline resistant *E. coli*, Enterobacteriaceae and total anaerobes. High abundances of *tet*(Q), *tet*(M), *tet*(W), *tet*(O) and *tet*(B) were detected in the inoculum and in the chemostat and did not significantly increase during administration of 1 and 4 mg l^{-1} DOX. Only 16 mg l^{-1} DOX caused minor enrichments. Subtherapeutic concentrations of doxycycline, which can be found in the feed as a result of cross-contamination, thus caused a selection pressure for resistant bacteria and negatively affected microbial activity, but did not influence to level of specific *tet* genes nor influenced the microbial community composition.

As a second part of Chapter 6, the microbial community composition and activity of the *in vitro* model, a chemostat simulating the cecal microbial ecosystem, was compared with the microbial communities in the pig's cecum. The *in vitro* ecosystem was characterized by a four-fold lower bacterial richness, as compared to its *in vivo* counterpart. Nevertheless, community profiling indicated that the *in vivo* and *in vitro* core communities were profoundly similar and also the VFA concentrations and compositions were comparable between *in vivo* and *in vitro* samples. This evaluation confirmed that the *in vitro* simulation provided an appropriate model to investigate the microbial ecosystem of the pig's cecum.

In general, this PhD thesis made use of multiple molecular and microbial research approaches to characterize the microbial communities associated with the rumen of cattle and the intestines of pigs. Metabarcoding was the common factor between these two divers research topics, but was supplemented by various other molecular and microbial analysis techniques. **Chapter 7** is therefore predominantly devoted to the discussion of the benefits and drawbacks of metabarcoding and exploring alternative options in the field of metagenomics.

SAMENVATTING

De vraag naar dierlijke producten is de afgelopen vijftig jaar meer dan verdriedubbeld vanwege de exponentieel groeiende wereldbevolking en de toegenomen welvaart. Om de stijgende vraag bij te houden, evolueerde de veeteeltsector naar intensieve productie systemen, gekarakteriseerd door een dichte bezetting van dieren, het voederen met energierijke diëten om groei en productie te bevorderen en het frequent toedienen van antibiotica om infecties te voorkomen of behandelen. Maar behalve een verhoogde productie, heeft intensive veeteelt ook een negatieve ecologische impact. De rundveehouderij heeft een enorm aandeel in de uitstoot van methaan, een potent broeikasgas dat wordt geproduceerd tijdens de spijsvertering. Daarnaast zorgt het frequente antibiotica gebruik in de veeteelt, onder andere in de varkenshouderij, voor de selectie en verspreiding van resistente bacteriën. Beide actuele problemen vinden hun oorsprong in de intestinale microbiële ecosystemen van deze nutsdieren.

Deze doctoraatsthesis omvat twee luiken: (1.) De identificatie van factoren die de bacteriële en methanogene gemeenschappen en activiteit in de pens van runderen sturen, en (2.) het onderzoek naar de invloed van kruisbesmetting van diervoeders met antibiotica op de microbiële gemeenschappen in de varkensdarm met doxycycline als voorbeeld. De overkoepelende doelstelling bij beide luiken was om metagenomics technieken te optimaliseren en toe te passen om belangrijke taxonomische groepen te identificeren, om populatie verschuivingen als reactie op interne of externe veranderingen in kaart te brengen en taxonomische veranderingen te linken aan functionaliteit.

Fundamentele kennis van het microbiële ecosysteem in de pens van runderen is essentieel in het onderzoek om productiviteit te verhogen en methaan uitstoot te verlagen. Het verzamelen van representatieve pensstalen staat centraal in deze studies, maar wordt bemoeilijkt door de complexiteit van het pens ecosysteem. De pens omvat immers verschillende microbiële habitats: de voedervezels, de pensvloeistof en het epitheel. Het doel van **Hoofdstuk 2** was om technieken te optimaliseren om de pensomgevingen afzonderlijk te kunnen bemonsteren, om verschillende staaltypen te evalueren op hun vermogen om het microbiële ecosysteem in de pens weer te geven en daarbij specifieke bacteriële en methanogene species te identificeren die eigen zijn aan de verschillende micro-omgevingen. Vijf staaltypen werden verzameld van vier gefistuleerde koeien: pensvezels (S), de geëlueerde species die gehecht zaten aan vezels (Ad),

vrijlevende species in de ruwe pensvloeistof (CRL), gezeefde pensvloeistof (Lq) en epithelium schraapsel (Ep). De resultaten geven aan dat voornamelijk de vloeibare en vezel-geassocieerde omgevingen werden onderscheiden door verschillen in abundantie van specifieke taxonomische groepen. Cellulolytische bacteriën als pionier van biofilm en secundaire biofilm-kolonisatoren waren dominant in vezelstalen (S en Ad), terwijl vloeistof-geassocieerde (CRL en Lq) species in de eerste plaats werden geïdentificeerd als consumenten van oplosbare nutriënten. Tevens bleek dat ook methanogene soorten een voorkeur hebben voor een ofwel vezel-geassocieerd ofwel vrij-levend voorkomen. Het epithelium werd gekenmerkt door een microbieel profiel dat afweek van deze van de vloeistof en vezelstalen. Tien bacteriële en twee methanogene families werden bijna uitsluitend in deze omgeving gevonden. Daarenboven vertegenwoordigt het CRL-staaltype het beste het globale microbiële ecosysteem van de pens, aangezien het zowel vezel-geassocieerde soorten omvat die loskomen uit mature biofilms als de bacteriën die een vrij-levend bestaan verkiezen.

Hoofdstuk 3 vertrekt van de hypothese dat ras-gerelateerde eigenschappen en opkweek strategien kunnen selecteren voor een ras-specifiek microbioom. De samenstelling van de microbiële gemeenschap en de methaanemissies van Holstein-Friesian (HF) melkvee en Belgisch Witblauw (DMBB) vleesvee werden vergeleken, terwijl andere factoren zoals voedersamenstelling, omgeving en fysiologie werden gestandaardiseerd. De methaanproductie (uitgedrukt in gram per dag) was significant hoger voor HF vaarzen in vergelijking met DMBB vaarzen, maar dit verschil werd niet weerhouden wanneer methaanopbrengst werd uitgedrukt per droge-stof opname. Hoewel het pens microbioom in beide rassen veel overkomsten vertoonden, bleken de bacteriële gemeenschappen in de pens van HF en DMBB vaarzen toch een ras-specifieke samenstelling te hebben, vanwege de differentiële abundantie van specifieke species die behoren tot de dominante taxonomische groepen en enkele (laag abundante) species die significant waren geassocieerd met een specifiek ras. In tegenstelling tot de bacteriën was de methanogene gemeenschap gelijkend en stabiel tussen rassen. Onze resultaten tonen dat ras-gerelateerde factoren de bacteriële samenstelling konden beïnvloeden, terwijl de variatie in methaanemissies voornamelijk kon worden toegeschreven aan de voederopname van de dieren.

Terwijl Hoofdstuk 3 de nadruk legt op de invloeden van ras op het pensmicrobioom, is **Hoofdstuk 4** gericht op het identificeren van de invloeden van de gastheer op de methanogene en bacteriële gemeenschappen in de pens. De invloed van de gastheer werd onderzocht door de samenstelling van de microbiële gemeenschappen van vier koeien te volgen na de transfer van de rumen inhoud van een donor koe naar drie acceptor koeien via de fistel, waarbij de invloed

van voedersamenstelling, omgeving, fysiologie werden geminimaliseerd. De donor koe werd geselecteerd uit vier gefistuleerde koeien (mid-lactatie) op basis van de hogere methaan productie. De pensinhoud van de donor werd grondig verwijderd en gebruikt als inoculum voor de lege pens van de donor zelf en drie acceptorkoeien. De koeien reageerden verschillend op deze verstoring van het pens ecosysteem. Direct na de transfer; de donor één van de acceptorkoeien leden een korte voeropname depressie, dat resulteerde in lagere methaanemissies, een andere vetzuur (VFA) samenstelling en een aangepast bacterieel profiel. De eerste twee dagen na de transfer nam de bacteriële rijkheid aanzienlijk af en kregen nieuwe taxa de kans om de gemeenschap te domineren. In de daaropvolgende dagen onderging de bacteriële gemeenschap verschillende autogene opvolgingen van bacteriën en werd een nieuwe stabiele steady-state bereikt. Daarentegen werd de voederopname en de fermentatie van de twee andere acceptorkoeien niet negatief beïnvloed door de pensinhoud transfer. Na de transfer namen de bacteriële gemeenschappen in de pens van deze acceptorkoeien initieel het profiel van de donor over, maar na enkele dagen bereikten de gemeenschappen een nieuw dynamisch evenwicht dat noch op het profiel van de donor noch op het oorspronkelijke profiel leek. Het bacteriële profiel blijkt dus niet louter gastheer afhankelijk maar wordt tevens beïnvloed door een waaier aan externe en interne factoren, waardoor het dynamisch is over tijd. De bacteriële gemeenschap bestond zodoende uit een kern van dominante en alomtegenwoordige species, aangevuld door een waaier aan minder dominante en transiënte bacteriën die elkaar opvolgden in tijd. De methanogenen daartegenover, werden gekenmerkt door een lage rijkheid en diversiteit maar werden niet beïnvloed door gastheer/interne/externe factoren.

In de hoofdstukken 2 tot en met 4 zijn experimenten beschreven waarbij metabarcoding werd gebruikt om het microbioom in de pens te karakteriseren en de factoren te identificeren die de taxonomische samenstellingen beïnvloeden. In het tweede luik van dit proefschrift werd het effect van lage dosissen doxycycline in varkensvoeder onderzocht, waarbij werd gefocust op het mogelijke effect op de bacteriële samenstelling van de intestinale gemeenschappen, hun metabolische activiteit en het aantal resistente bacteriën en resistentie genen.

Een preliminair *in vivo* experiment (**Hoofdstuk 5**), uitgevoerd op het CODA, bepaalde de intestinale en fecale concentraties van antibiotica wanneer varkens een dieet kregen met residuen van een antibioticum door kruiscontaminatie, d.w.z. dat het 3% van de maximale aanbevolen concentratie van het specifieke antibioticum bevatte. Doxycycline (DOX), een semisynthetisch antibioticum van de tetracyclinegroep, bereikte een stabiele concentratie van ongeveer 4 mg kg^{-1} in de mest van behandelde varkens na vier dagen het met antibiotica

gesupplementeerde voeder dieet toe te dienen. Tegelijkertijd namen de tetracycline-resistentiegenen *tet*(W) en *tet*(L) significant toe, terwijl de abundanties van andere geteste resistentiegenen *tet*(O), *tet*(Q), *tet*(A), *tet*(M), *tet*(B) niet significant stegen tijdens behandeling. De microbiële gemeenschappen in de varkensmest werden niet beïnvloed door de continue toediening van subtherapeutische doxycycline, aangezien geen enkele taxonomische groep significant af -of toenam tijdens behandeling met DOX, in vergelijking met de controlegroep. Er werden slechts korte-termijn effecten waargenomen op de microbiële rijkheid en diversiteit, die het laagst was op de vierde dag van toediening. Zodoende zorgde de versleping van 3% van een therapeutische dosis DOX voor de aanrijking van slechts enkele tetracycline resistentiegenen, maar had geen invloed op de samenstelling van de fecale microbiële gemeenschappen van varkens.

De experimenten in **Hoofdstuk 6** onderzochten de effecten van deze gemeten intestinale concentraties aan doxycycline op het intestinale microbiële ecosysteem. Naast de concentraties gevonden in de darmen als gevolg van kruisbesmetting, d.w.z. 1 en 4 mg DOX per liter voedingsmedium, werd ook het effect van een referentieconcentratie van 16 mg l^{-1} onderzocht. Deze concentraties werden continu toegediend aan een chemostaat, die het microbiële ecosysteem van de blinde darm (i.e. het cecum) simuleert en werd geïnoculeerd met de caecale inhoud van biologisch gekweekte varkens. De toediening van zelfs de laagste concentratie DOX veroorzaakte een significante afname in bacteriële activiteit, desondanks had geen van de geteste concentraties een observeerbare invloed op de microbiële gemeenschap. De bacteriële gemeenschappen in het caecum van biologische varkens (ondanks dat deze geen antibioticum behandelingen kregen) omvatten reeds hoge concentraties van specifieke tetracycline gene *tet*(Q), *tet*(M), *tet*(W), *tet*(O)en *tet*(B) en in de chemostaat (voor DOX toediening) werden deze concentraties behouden, ondanks de selectiedruk voor snelgroeiende species. De aantallen van deze tetracycline resistentiegenen namen niet significant toe tijdens de toediening van 1 en 4 mg l^{-1} DOX. Slechts 16 mg l^{-1} DOX veroorzaakte kleine vermeerdering. In tegenstelling tot de beperkte effecten van de DOX concentraties op de abundanties van de geteste resistentie genen (a.d.h.v. qPCR assays), toonde uitplatingen een duidelijke impact van subtherapeutische DOX concentraties op de aantallen van resistente bacteriën. Zelfs de laagste concentratie (1 mg l^{-1} DOX) veroorzaakte een selectiedruk voor tetracycline resistente *E. coli* maar niet voor andere taxonomische groepen. Daarentegen zorgde 4 mg l^{-1} voor een significante aanrijking van tetracycline-resistente *E. coli*, Enterobacteriaceae en totale anaeroben. Subtherapeutische DOX concentraties, die in het voeder terecht kunnen komen als gevolg van kruisbesmetting,

selecteerden voor tetracycline resistente bacteriën en verlaagden de microbiële activiteit, maar hadden echter geen invloed op de abundantie van specifieke tet-genen en hadden geen invloed op de samenstelling van de microbiële gemeenschap wanneer gekeken werd via metabarcoding maar dus wel wanneer klassieke uitplating uitgevoerd werden.

Een tweede onderzoeksdoel van Hoofdstuk 6 het vergelijken van de samenstelling en activiteit van de microbiële gemeenschap van het *in vitro* model (een reactorsysteem dat het cecale microbiële ecosysteem simuleert) met de microbiële gemeenschappen in het cecum van varkens. Het *in vitro* microbiële ecosysteem werd gekenmerkt door een vier keer lagere bacteriële rijkheid, in vergelijking met het *in vivo* ecosysteem vertegenwoordigd door cecale stalen. Desalniettemin toonde de ordinatie van de stalen dat de *in vivo* en *in vitro* microbiële gemeenschappen gelijkend waren in taxonomische samenstelling en abundanties. Vetzuur profielen bewezen tevens dat ook de metabolische activiteit van de bacteriën in de *in vivo* en *in vitro* omgeving overeen kwamen. Deze evaluatie bevestigde dat de *in vitro* simulatie een geschikt model was om het microbiële ecosysteem van het varkenscecum te onderzoeken.

In dit proefschrift werd in beide onderzoeksluiken gebruik gemaakt van meerdere moleculaire en microbiële onderzoekstechnieken om de microbiële gemeenschappen geassocieerd met enerzijds de pens van rundvee en anderzijds de varkensdarm te karakteriseren. Metabarcoding was de gemeenschappelijke factor tussen deze twee verschillende onderzoeksthema's, maar werd aangevuld met diverse andere moleculaire en microbiële analysetechnieken. Hoofdstuk 7 is daarom voornamelijk gewijd aan de bespreking van de voor- en nadelen van metabarcoding en het beschrijven van alternatieve opties op het gebied van metagenomics.

Scientific curriculum

SCIENTIFIC CURRICULUM

Personalia

Name	Thijs De Mulder
Address	H. Frere Orbanlaan 292, 9000 Ghent (Belgium)
Telephone	+32 494 89 62 88
E-mail	thijs.demulder@yahoo.com
Date of birth	July 29, 1990
Place of birth	Ghent
Nationality	Belgian

Education

2002-2008	Science-Mathematics Sint-Franciscusinstituut, Melle
2009-2013	Master of Bioscience Engineering: Cell and Gene Biotechnology Faculty of Bioscience Engineering, Ghent University Thesis: the influence of the mucin degrading species *Akkermansia muciniphila* on the colon microbiota and the health status of the host. (CMET, Ghent University)
2013-2017	PhD research CMET, Faculty of Bioscience Engineering, Ghent University Technology and Food Science Unit, LVO Animal Science Unit, ILVO

De Mulder, T., Goossens, K., Peiren, N., Vandaele, L., Haegeman, A., De Tender, C., Ruttink, T., Van de Wiele, T. and De Campeneere, S. Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. FEMS *Microbiology Ecology*. 2016, fiw251.

De Mulder, T., Peiren, N., Vandaele, L., Ruttink, T., De Campeneere, S., Van de Wiele, T. and Goossens, K. Impact of breed on the rumen microbial community composition and methane emission of Holstein Friesian and Belgian Blue heifers. *Livestock Science*. 2017.

De Mulder, T., Rasschaert, G., Van Coillie, E., Van den Meersche, T., Ruttink, T., Van de Wiele, T. and Heyndrickx, M. Impact of subtherapeutic concentrations of doxycycline hyclate on the microbial ecosystem in an *ex vivo* model of the pig's cecum. *J. of Applied Microbiology*. (revisions submitted)

De Mulder, T., Vandaele, L., Peiren, N., Haegeman, A., Ruttink, T., De Campeneere, S., Van de Wiele, T. and Goossens, K. Host influences and dynamic fluctuations of the bacterial and methanogen community following a complete ruminal content transfer. *J. of Agricultural Science*. (under review)

Peeters, L.E.J., **De Mulder, T.**, Van Coillie, E., Huygens, J., Smet, A., Daeseleire, E., Dewulf, J., Imberechts, H., Butaye, P., Haesebrouck, F., Croubels, S. and Heyndrickx, M. Selection and transfer of an IncI1- tet (A) plasmid of Escherichia coli in an ex vivo model of the porcine caecum at doxycycline concentrations caused by crosscontaminated feed. *J. of Applied Microbiology*. 2017, 123, 1312–1320.

Calliauw, F., **De Mulder, T.**, Broekaert, K., Vlaemynck, G., Michiels, C. and Heyndrickx, M. Assessment throughout a whole fishing year of the dominant microbiota of peeled brown shrimp (Crangon crangon) stored for 7 days under modified atmosphere packaging at 4 °C without preservatives. *Food Microbiology*. 2016, 54, 60–71.

Daeseleire, E., De Graef, E., Rasschaert, G., **De Mulder, T.**, Van den Meersche, T., Van Coillie, E., Dewulf, J. and Heyndrickx, M. Antibiotic use and resistance in animals: Belgian initiatives. *Drug Testing and Analysis*. 2016, 8, 549–555.

Van Herreweghen, F., Van den Abbeele, P., **De Mulder, T.**, De Weirdt, R., Geirnaert, A., Hernandez-Sanabria, E., Vilchez-Vargas, R., Jauregui, R., Pieper, D. H., Belzer, C., De Vos, W. M. and Van de Wiele, T. In vitro colonisation of the distal colon by Akkermansia muciniphila is largely mucin and pH dependent. *Beneficial Microbes*. 2017, 8, 81–96.

Conferences, symposia and workshops

- 20th Conference on Food Microbiology (BSFM), Brussels, Belgium. October 8-9, 2015.

  De Mulder, T., Robyn, J., Rasschaert, G., Van Coillie, E., Van de Wiele, T. and Heyndrickx, M. Impact of cross-contamination of feed with antimicrobial compounds on the microbial population in the gastr-intestinal tract of pigs. **Poster presentation**.

- BIG N2N annual symposium, Ghent, Belgium. May 21, 2015.

  Haegeman, A., **De Mulder, T.**, De Tender, C., et al. Towards populations genomics and metagenomics in agriculture and marine environments. **Conference paper**.

- RuminOmics Final Conference, Paris, France. December 7, 2015

- INRA-ROWETT Symposium on Gut Microbiology, Clermont-Ferrand, France. June 20-23, 2016.

  De Mulder, T., Goossens, K., Van de Wiele, T. and De Campeneere, S. Exploring the methanogen and bacterial communities of rumen environments. **Poster presentation**.

- 6th Greenhouse Gas & Animal Agriculture Conference (GGAA), Melbourne, Australia. February 14-18, 2016.

  De Mulder, T., Goossens, K., Peiren, N., Vandaele, L., Van de Wiele, T. and De Campeneere, S. Influence of host-dependent factors on the methanogen community and methane production. **Poster presentation**.

- 21th Conference on Food Microbiology (BSFM), Brussels, Belgium. September 15-16, 2016.

  De Mulder, T., Rasschaert, G., Van Coillie, E., Van de Wiele, T. and Heyndrickx, M. Effect of three concentrations of doxycycline on the microbial community and resistance development in the *ex vivo* pig gut. **Oral presentation**.

Workshops and courses

- Seminar "Getting started with High-Performance Computing" (Unix command line, Python scripting and HPC basics). Prof. P. Dawyndt. Ghent University, Ghent, Belgium. May 26-28 and June 2-4, 2014.

- Training: “Arctic Microbiology: education and training in field work and analysis” Prof. M. Höfte. University of Akureyri, Akureyri, Iceland. June 16-29, 2014.
- Course: “MG-RASR”. Dr. Folker Meyer. Ghent University, Ghent, Belgium. September 17-18, 2014
- Course: “Linux for bioinformatics”. VIB-BITS, Ghent, Belgium November 14, 17, 2014.
- Course : “Laboratory Animal Science, module 1 and 2”. Ghent University, Merelbeke, Belgium. September-November, 2014.
- Course: “English Presentation Skills”. Ghent University, Ghent, Belgium. Februari 15 – March 15, 2017.

Dankwoord

DANKWOORD

Nu de laatste hand aan mijn thesis is gelegd, neem ik de tijd om terug te blikken naar de voorbije jaren en stil te staan bij alle mensen die bewust of onbewust hebben bijgedragen tot de realisatie van dit doctoraat.

In de eerste plaats ben ik zeer dankbaar voor de kans die ik heb gekregen om te doctoreren op het ILVO, onder het promotorschap van Tom, Marc, Els en Sam, die later de fakkel doorgaven aan Tom, Geertrui en Karen. Bedankt om mij bij te staan tijdens de laatste vier jaar, voor de waardevolle ideeën, het geduld en de enthousiaste begeleiding! Tijdens onze vele vergaderingen gaven jullie mij nieuwe ideeën en inzichten om de experimenten uit te voeren en data te analyseren. Daarnaast kon ik ook altijd binnenspringen voor praktische en wetenschappelijke vragen.

Tom en Annelies, jullie hebben veel tijd geïnvesteerd in mijn project door mee te denken over dataverwerking en visualisatie, protocollen en pipelines te ontwikkelen, en nog zoveel meer. Gelukkig was ik niet de enige op een Genomics-doctoraat maar werd ik vergezeld door drie andere doctorandi: Caroline, Christophe en Elisabeth. Bedankt voor de noodzakelijke ontspanning na de werkuren, hopelijk kunnen we onze BBQ traditie nog lang voortzetten!

Toen ik startte aan mijn doctoraat zaten er nog grote hiaten in mijn kennis over *in vivo* experimenten zoals voedersamenstelling en huisvesting. Gelukkig kon ik altijd ten rade gaan bij Karen, Leen, Nico en Sam om mij te helpen bij de proefplanning, staalname en dataverwerking. Stefaan, Jorne, Michaël en Sara, ook jullie hebben mij in grote mate geholpen tijdens de praktische zaken van de dierexperimenten. Bedankt voor de aangename samenwerking en vooral om de koeien in bedwang te houden tijdens de vele staalnames, te helpen bij de GUKs en mij te leren melken. Ook mijn bureaugenoten wil ik niet vergeten: Sieglinde, Elisabeth, Dorien en Pieter, bedankt voor de leuke (niet-werk gerelateerde) conversaties, de vele second-opinions en de aangename bureausfeer!

Ook op T&V heb ik kunnen genieten van een zeer aangename werksfeer tussen de collega's. Bedankt Helder, Sharon, Tina, Niels, Thomas, Bavo, Kaas, Lien, Nikki, Laura, Marijke en Ann, voor de gezellige middagpauzes en de leuke sfeer in het labo!

Als voorlaatste zou ik graag nog de niet-werk gerelateerde vrienden bedanken voor de leuke reizen en de ontspanningsmomentjes! Zodoende, bedankt aan Kim, Britts, Gio, Kwirsten, Tinfong, Tom, Kristof, Liselot, Jelle, Sofie, Francis, Benjamin, Jelke, Nelleke en Tess!

De laatste paar regels heb ik voorbehouden voor de mensen die de grootste drijfveer vormen achter alles wat ik reeds heb bereikt. Mijn teerbeminde papa en mama die me van bij het begin als prille bio-ingenieur tot doctor hebben bijgestaan. Bedankt voor jullie steun, geduld, hulpvaardigheid en interesse in mijn onderzoek... Zonder jullie zou het niet gelukt zijn! Ook een dikke merci aan mijn lieftallige zus Blekes die er ook op haar manier bij betrokken was.

Ten slotte zou ik graag ook Evelien bedanken, die nu naast mij zit terwijl ik dit schrijf dus zal ik er maar best iets liefs van maken… Bedankt dat ik altijd bij jou kan thuiskomen, dat jij mijn stress wegneemt, en voor alle kleine dingen die je voor mij en voor dit doctoraat hebt gedaan!