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Gut metagenomics in relation to diet and methanogenesis in arctic herbivores

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Table of contents

1. List of papers

This thesis is based on three papers referred to in the text by a Roman numeral as follows:

I. Salgado-Flores A, Hagen LH, Ishaq SL, Zamanzadeh M, Wright AD, Pope PB, Sundset MA. Rumen and Cecum Microbiomes in Reindeer (*Rangifer tarandus tarandus*) Are Changed in Response to a Lichen Diet and May Affect Enteric Methane Emissions. PLoS ONE. 2016; 11: e0155213.

II. Salgado-Flores A, Bockwoldt M, Hagen LH, Pope PB, Sundset MA. First insight into the faecal microbiota of the high Arctic muskoxen (*Ovibos moschatus*). Microbial Genomics. 2016; DOI: 10.1099/mgen.0.000066.

III. Salgado-Flores A, Tveit AT, Wright AD, Pope PB, Sundset MA. Characterization of the cecum microbiome from wild and captive rock ptarmigans from Svalbard and northern Norway in relation to diet composition. Manuscript.

2. Abbreviations

CH_4: methane

H_2: hydrogen

PSMs: plant secondary metabolites

qRT-PCR: quantitative real-time polymerase-chain reaction

SGMT-methanogens: *Methanobrevibacter smithii-Metanobrevibacter gottschalki-Methanobrevibacter millerae-Methanobrevibacter-thaueri*

RO-methanogens: *Methanobrevibacter ruminantium-Methanobrevibacter olleyae*

GHs: glycoside hydrolases

VFAs: volatile fatty acids

CO_2: carbon dioxide

GE: gross energy

GHG: greenhouse gases

3. Thesis abstract

Enteric methane (CH_4) resulting from the microbial fermentation of complex organic polymers, is produced by a specialized group of microbes called methanogenic Archaea – or methanogens. Methanogens play an important role in anaerobic fermentation by removing hydrogen (H_2) as its accumulation may lead to the disruption of these anaerobic processes. The resulting production of CH_4 may constitute a substantial loss of energy for the animal host, varying with diet. In addition, CH_4 released by herbivores also accounts for a substantial fraction of the global anthropogenic CH_4 emissions (≈22%), and big efforts have been put into its reduction. Extensive research exists on enteric CH_4 production and methanogens in domestic ruminants, but little is known with regard to arctic herbivores.

This PhD thesis aims at improving our understanding of the gut microbiome in three arctic herbivores, Norwegian reindeer (*Rangifer tarandus tarandus*), muskox (*Ovibos moschatus*), and rock ptarmigan (*Lagopus muta*), with special emphasis on CH_4 metabolism. Investigating the microbiological bases of methanogenesis is of great interest and allows a more detailed understanding of their digestive physiology, and by extension of the whole-body physiology of these arctic animals. They typically consume plants rich in toxic plant secondary metabolites (PSMs), which, in high concentrations, may depress enteric CH_4 production. The objectives of this thesis were 1) to investigate the influence of diet composition (especially PSMs) on the gut microbiota in these arctic herbivores, and its relationship with methanogenesis; 2) to characterize specific methanogens linked to low CH_4 potentials, which might be useful for the development of strategies focused on reducing enteric CH_4, especially in ruminants; and 3) to describe for the first time the gut microbiome in muskoxen and wild rock ptarmigans (from Svalbard and northern Norway) in order to figure out the ecological role played by the gut microbiota in these animals.

Three different platforms were applied based on our specific goals: (1) Quantitative real-time PCR (qRT-PCR) was used for the quantification of microbial groups (methanogens, bacteria, and protozoa) (**PAPER I and III**); (2) Amplicon 16S rRNA sequencing (ROCHE 454 pyrosequencing Titanium technology) was chosen for the taxonomical characterization of the major archaeal and bacterial phylotypes (**PAPER I, II and III**); and (3) shotgun metagenomics (Illumina HiSeq 3/4000) was applied to get an overall picture of the genetic information related to this microbiota (**PAPER III**).

The archaeal and bacterial profiles were significantly different between reindeer fed lichens (high in PSMs) and pellets concentrate (**PAPER I**). When methanogens were classified into two

major groups based on their phylogenetic relatedness, namely, SGMT-methanogens (*M. smithii, M. gottschalki, M. millerae, M. thaueri*) or RO-methanogens (*M. ruminantium, M. olleyae*), the ratio between these two clades (SGMT:RO) was lower in Norwegian reindeer fed lichens due to an increase of the RO-group. A low SGMT:RO ratio was also found in the feces of muskoxen grazing on graminoids (**PAPER II**). Previous CH_4 records from these wild ruminant species were relatively low compared to domestic ruminants. These findings may indicate that increased relative abundance of RO-methanogens (thus decreasing the SGMT:RO ratio) may be linked to the low CH_4 yields. Lichens are naturally ingested by Norwegian reindeer mainly in winter and muskoxen feces for this study were collected during fall, thus the situation described by this work relates to such periods. In particular to Norwegian reindeer, a possible interpretation of these findings is that housing methanogens with expected low CH_4 potentials might help reduce the energy lost as CH_4 in periods with austere nutritional conditions.

Methanol-utilizing *Methanomassiliicoccaceae* (**PAPER III**) were dominant in the cecum of wild ptarmigans fed their respective natural diets (*Salix* spp., *Betula* spp., and *Empetrum* spp.), and their abundance was related to methanol production via bacterial pectin degradation although a specific link between dominance of *Methanomassiliicoccaceae* and low CH_4 emission could not be established.

Muskoxen presented fecal bacterial profiles dominated by *Firmicutes,* mostly related to the fibrolytic family *Ruminococcaceae* (**PAPER II**), reflecting their highly fibrous autumn diet. Wild rock ptarmigans housed a diverse microbiota with bacterial groups involved in the degradation of PSMs (e.g. *Firmicutes, Bacteroides, Synergistetes,* etc) (**PAPER III**). This microbiota also presented a wide range of hydrolytic enzymes involved in the degradation of hemicellulose and non-cellulosic polysaccharides (pectin, starch). It would allow ptarmigans to feed on plants with variable fiber contents despite their high PSMs contents, which might be an advantageous strategy in periods with low availability of high-quality food.

This PhD project also shows the complexity of relating specific archaeal and bacterial profiles to CH_4 potential. Our findings suggest that other factors apart from (or in addition to) the dietary PSMs contents (e.g. type of polysaccharides) may influence the taxonomy of methanogens, and therefore methanogenesis. Finally, the insights on the microbiota from muskoxen and wild rock ptarmigans allowed a better understanding on the digestive physiology in these two arctic herbivores as well as the role played by the gut microbiota in the adaptation to their respective diets.

4. Aims

This PhD thesis aims at expanding our understanding of the gut microbiome in three arctic herbivores: Norwegian reindeer (*Rangifer tarandus tarandus*), muskox (*Ovibos moschatus*), and rock ptarmigan (*Lagopus muta*), in relation to CH_4 metabolism. Enteric CH_4 may result in an energy loss for these ruminants, which can be important under austere nutritional conditions with limited energy supply opportunities. Investigating the microbiology of methanogenesis in these animals would help obtain a more detailed understanding of their digestive physiology, and by extension of their whole-body physiology. Norwegian reindeer, muskox and ptarmigan naturally consume diets with high contents of plant secondary metabolites (PSMs), whose ingestion may depress enteric methanogenesis. The main goals of this thesis were 1) to investigate the influence of diet composition (especially PSMs) on the gut microbiota in these Arctic herbivores, and its relationship with methanogenesis; 2) to characterize specific methanogens linked to low CH_4 potentials, which may be useful for the development of strategies focused on reducing enteric CH_4, especially in ruminants; and 3) to describe for the first time the gut microbiome in muskoxen and wild rock ptarmigans (from Svalbard and northern Norway) in order to figure out the ecological role played by the gut microbiota in these animals.

Overall hypotheses:

- The ingestion of diets rich in PSMs by these arctic herbivores may lead to the presence of specific methanogens that may account for their reported low CH_4 outputs.

- Muskoxen and rock ptarmigans possess a gut microbiota specialized in the degradation of their respective natural diets.

5. Introduction

5.1. The gut microbiota

Herbivores are unable to synthesize the enzymatic machinery necessary to degrade the complex mesh of polysaccharides constituting the ingested plant materials, so they must rely on a symbiotic microbiota to perform this task. This microbial consortium is constituted by several microbial groups, both prokaryotic and eukaryotic, namely, bacteria, methanogenic archaea –or methanogens-, protozoa (mostly ciliated) and fungi, coexisting harmonically in their gastrointestinal tract occupying a specific niche according to their physiological traits [1, 2].

5.1.1. Prokaryotic microbiota

The gut microbiota in ruminant and non-ruminant herbivores have been extensively characterized [3-6]. Bacteria constitutes more than 95% of the total microbial community in the rumen, and despite their broad diversity they are mostly associated to a few phyla such as *Bacteroidetes, Firmicutes* and *Fibrobacteres* (Table 1) [1]. Several factors such as diet composition, pH, age, or the site across the digestive tract may influence bacterial taxonomy [7,8]. The different bacteria possess specific physiological characteristics: some bacteria are specialized in the degradation of complex polysaccharides such as cellulose or hemicellulose, whereas others may degrade soluble compounds (e.g. starch). Rumen bacteria can be subdivided according to their physical niche [9]: 1) bacteria attached to the feed particles (approximately 75%); 2) planktonic bacteria found in the rumen liquor; 3) bacteria attached to the rumen epithelium; 4) and bacteria attached to the surface of eukaryotic microbes (protozoa and fungi). Accordingly, the bacterial taxonomy would vary based on their specific niche [10], but instead of acting separately bacteria from these different locations interact with each other forming a complex community.

The taxonomy of methanogens from herbivores has also been extensively investigated, mostly focused on domesticated ruminants (Table 2). In general, *Methanobrevibacter* was the dominant genus, displaying a greater diversity at species level mostly influenced by diet composition, with other genera are also present but at a minor proportion. A more detailed description of the different type of methanogens as well as their respective metabolic pathways is given in section 2.3.

Table 1. Major bacterial groups described in different ruminant and non-ruminant herbivores.

Animal Source	Diet	Predominant bacteria (% total sequences)	Ref.
Norwegian reindeer (*Rangifer tarandus tarandus*)*	Natural summer pasture Pelleted concentrate	*Bacteroidales* (29%); *Clostridiales* (71%) *Clostridiales* (91%)	[11]
Svalbard reindeer (*Rangifer tarandus platyrhyncus*)*	Natural late summer pasture Natural winter pasture	*Bacteroidales* (42%); *Clostridiales* (55%) *Bacteroidetes* (60%); *Firmicutes* (27%)	[11] [12]
Sika deer (*Cervus nippon*)*	Corn, soybean, grains + oak leaves Corn, soybean, grains + corn stalks Corn, soybean, grains + corn silage	*Prevotella* (45%); *Succinivibrio* (9%) *Prevotella* (57%); *Succinivibrio* (13%) *Prevotella* (50%); *Succinivibrio* (10%)	[13]
Beef steer (*Bos taurus*)*	Prairie hay:concentrate (80:20) Prairie hay:concentrate (20:80)	*Firmicutes* (33%); Unclassified (31%); *Bacteroidetes* (22%) *Bacteroidetes* (44%); *Firmicutes* (39%); Unclassified (10%)	[14]
Beef steer (*Bos taurus*)°	Grain(corn):silage / hay (66:26) Grain(corn):silage / hay (83:13) Grain(corn):silage / hay (0:100)	*Ruminococcaceae* (15%); *Lachnospiraceae* (13%) *Prevotella* (15%); *Lachnospiraceae* (13%) *Ruminococcaceae* (33%); *TM7* (11%)	[15]
Korean goat (*Capra aegagrus coreanae*)°	Concentrate:Bermuda grass (90:10)	*Bacteroidetes* (50-70%); *Firmicutes* (20-40%)	[16]
Horse (*Equus caballus*)°	Pelleted concentrate	*Streptococcus* (24%); *Verrucomicrobia* (17%); *Clostridiaceae* (10%)	[17]
Japanese rock ptarmigan (*Lagopus muta japonica*) (wild)°	Leaves *Empetrum nigrum*, *Rhododendron*, Japanese stone pine	*Coriobacteriaceae* (17%); *Synergistaceae* (11%); *Bacteroides*	[18]
Svalbard rock ptarmigan (captive)°	Pelleted concentrate, leaves	*Ruminococcaecae* (25%); *Clostridiales* (18%); *Ruminococcus* (11%)	[18]

*Rumen / crop samples.
° Cecum / fecal samples.

Table 2. Major archaeal groups described in different ruminant and non-ruminant herbivores

Animal Source	Diet	Predominant methanogens (% total sequences)	Ref.
Holstein cow (*Bos taurus*)*	High fiber diet	*Methanobrevibacter ruminantium* (50%); *Methanobrevibacter millerae* (27%)	[19]
Jersey cow (*Bos taurus*)*	High fiber diet	*M. ruminantium* (31%); *M.millerae* (48%)	[19]
Hereford cattle (*Bos taurus*)* Hereford cattle*	Corn-based diet Potato byproducts diet	*M. ruminantium* (48%); Unclassified *Thermoplasma* (38%) Unclassified *Thermoplasma* (50%); *M. ruminantium* (21%); *Methanobrevibacter smithii* (19%)	[20]
Norwegian reindeer (*Rangifer tarandus tarandus*)*	Natural summer pasture	*M. ruminantium* (31%); *Methanobrevibacter gottschalkii* (28%); *M. smithii* (24%)	[3]
Svalbard reindeer (*Rangifer tarandus platyrhynchus*)	Late fall pasture	*Aciduliprofundum boonei* (49%); *M. millerae* (28%)	[21]
Impala (*Aepyceros melampus melampus*)*	Natural pastures	*Methanobrevibacter thaueri* (51%); *M. smithii* (27%)	[22]
Sika deer (*Cervus nippon*)*	Pelleted concentrate + oak leaves Pelleted concentrate + corn stalks	*M. millerae* (51%); *Methanobrevibacter wolinii* (34%) *M. millerae* (78%); *Methanomassiliicoccus luminyensis* (9%)	[23]
Yak (*Bos grunniens*)*	Kobresia pasture	Unclassified *Mms. luminyensis* (52%); *M.millerae* (7%)	[24]
Chinese cattle (*Bos taurus*)*	Kobresia pasture	Unclassified *Mms. luminyensis* (62%); *M. millerae* (17%)	[24]
Moose (*Alces alces*, Norway)*	Natural Fall diet	*M. ruminantium* (41%); *Methanosphaera Stadtmanae* (17%); *M. thaueri* (13%)	[5]
Bactrian camel (*Camelus bactrianus*)°	Hay + alfalfa (high fiber) Hay + starch concentrates	*M. millerae/M. thaueri/M. gottschalkii* (78%); *M. ruminantium* (18%) *M. ruminantium* (58%); *M. millerae/M. thaueri/M. gottschalkii* (21%)	[25]
Hanwoo (*Bos taurus coreanae*)°	Concentrate + rice straw (rumen fluid) Concentrate + rice straw (rectal dung)	*M. ruminantium* (63%); *M. millerae* (21%) *Methanocorpusculum labreanum* (53%); *M. millerae* (33%)	[26]

White rhinoceroses (*Ceratotherium simum*)°	Pellets, apple, carrot, fresh forage/alfalfa, alfalfa hay (10:5:10:80:10)	*Mcp. labreanum* (60%); *M. smithii* (27%)	[27]
Hoatzin (*Opisthocomus hoazin*)*	Natural diet	*M. ruminantium* (84%); Unclassified *Mbb. stadtmanae* (14%)	[28]
Adult chicken (*Gallus gallus domesticus*)°	Layer ration diet (*Donalson et al.* 2005)	*Mbb. woesei* (99%)	[29]

*Rumen / crop samples.
° Cecum / fecal samples.

5.1.2. Eukaryotic microbiota

Eukaryotic microorganisms like protozoa and fungi also contribute to the anaerobic degradation of the various polysaccharides from the ingested material. Ciliates, the major group of protozoa in the digestive tract of hebivores, may constitute up to 50% of the total biomass in the rumen and account for up to one-third of the total fiber degradation [30,31]. The exact mechanism whereby ciliates carry out the degradation of fiber remains unclear, but genes for putative hydrolytic enzymes mediating the degradation of polysaccharides have been characterized [32,33]. Rumen ciliates are mainly classified into two major orders, Entodiniomorphids and Vestibuliferida, and mostly associated to the genera *Entodinium, Polyplastron, Eudiplodinium,* e *Isotricha,* among others [3,34]. Symbiotic associations between some ciliates and methanogens in gut environments have been described, providing the former with H_2 and shelter to methanogens [35,36].

Anaerobic fungi may account for 5% up to 20% of the rumen biomass, and are mainly involved in degradation of cell wall polysaccharides and the fermentation of the resulting byproducts [37]. Similarly to ciliates, these microorganisms also synthesize a wide array of hydrolytic enzymes, enabling them to penetrate plant cell walls and get access to fermentable substrates used for their metabolism [9,38]. Further colonization by gut fungi of plant particles leads to their disruption into smaller particles and favours their degradation by the other syntrophic microbes. Anaerobic fungi are constituted by a monophyletic clade (*Neocallimastygomycetes*) separated from basal fungi, only one family, and six genera in total [39].

5.2. Anaerobic processes involved in food degradation

5.2.1. Types of plant polysaccharides and their degradation

The cell wall of plants consists mainly of various types of plant polymers such as fibrous (cellulose, hemicellulose and lignin) and non-fibrous compounds (starch, pectin) intermeshed and chemically bond to one another by covalent and non-covalent cross-linkages (Fig. 1) [40]. Due to their heterogeneous composition, cell wall-degrading microorganisms produce a battery of enzymes, named glycoside hydrolases (GHs), mediating the hydrolysis of the glycosidic bond existing between monosaccharides leading to their catabolism (http://www.cazy.org/) [41,42]. Numerous GH familes exist, each of them with particular substrate specificity, sharing conserved catalytic apparatus and mechanisms of action [41]. There also exist other enzymes with hydrolytic or structural properties intervening in the degradation of polysaccharides such as polyssacharide lyases (PLs), carbohydate esterases (CEs), and carbohydrate-binding modules (CBMs).

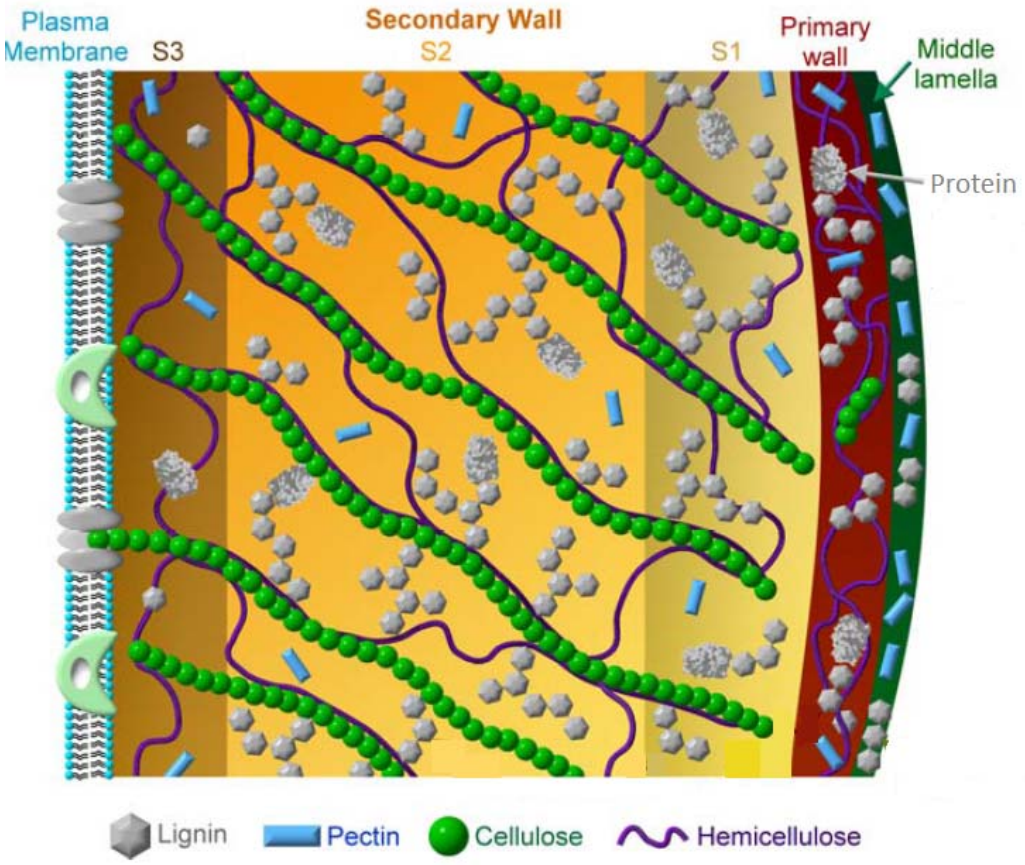

Fig 1. Illustration of a 'typical' plant cell wall and their main structural components. The relative thickness for the different layers is also showed. The different symbols represents the various structural components: lignin, hemicellulose, cellulose, pectin and proteins. Modified from [147]. Reprinted with permission.

Many bacteria synthesize and release an enzymatic machinery in the space between the plant particle and the microbe (cellulosome) consisting of a wide range of hydrolytic and structural proteins [2,43]. Other groups like *Bacteroidetes* contain gene clusters, named polysaccharide utilization loci (PULs), encoding a suite of proteins involved in the depolymerization of specific substrates [44].

Cellulose is a linear homopolymer constituted of D-glucose molecules linked by *β*-1,4-glycosidic bonds. Cellulases are GHs hydrolyzing the *β*-1,4-glycosidic bonds of cellulose molecule. First, exo-1,4- *β*-glucanases degrades the cellulase chains into oligosaccharides, then cleaved into mono- or disaccharides by endo-1,4- *β*-glucanases. β-glucosidases further cleaves off the resulting cellobiose molecules into two glucoses [40]. Several bacterial groups (genera *Ruminococcus*, *Clostridium* and *Fibrobacter*), fungi and protozoa showed cellulose-degrading activity [2,38,45].

Hemicellulose is constituted by several types of sugars arranged in a more heterogeneous distribution than cellulose, whose degradation requires the cooperative action of several

enzymes (e.g. endo-1,4-*β*-xylanases, xylan 1,4-*β*-xylosidases, for xylan). Debranching enzymes (e.g. acetyl esterases, arabinofuranosidases) cleave off side-chain monosaccharides whereas other set of enzymes degrades the inner molecules. Degradation of hemicellulose have been reported to bacteria from *Fibrobacter, Ruminococcus, Prevotella* and *Butyrivibrio* [46].

Lignin are non-soluble organic biopolymer possessing a complex chemical architecture constituted by phenylpropanoids units linked via –O- (ether) or C-C bonds [47]. Under methanogenic conditions, lignin degradation requires the cooperative action of various microbial groups such as acetogens, ring-cleaving fermenters and acetate/H_2-consuming methanogens [48].

Pectin are non-fiber polysaccharides primarily found as part of the middle lamella joining the cell wall of two cells. In general, they possess a chemical composition mainly constituted by galacturonans and rhamnogalacturonans bond with α-1,4- glycosidic linkages, and neutral sugars as side chains [49,50]. Pectin degradation are catalyzed by pectinases (pectolyase, pectozyme and polygalacturonases), which cleave off the linkages between molecules of pectin [51]. *In vitro* studies showed pectin-degradation by bacteria belonging to the genera *Prevotella* and *Treponema* in ruminal fluid and fecal samples [52,53].

5.2.2. Primary fermentation

The anaerobic degradation of complex organic polymers (e.g. cellulose) into carbon dioxide (CO_2) and CH_4 is characteristic of environments depleted of electron acceptors such as O_2, nitrate, sulfate, etc., as occurs in the intestinal tract of animals. These anaerobic processes require the co-participation of several microbial groups (Fig. 2) [54]. Oligomers (e.g. sugars, amino acids) from the hydrolysis of plant polymers are converted into volatile fatty acids (VFAs) (mostly butyrate, propionate, formate), alcohols, lactate, H_2 and CO_2 (primary fermentation). Alternatively, part of the resulting acetate, H_2, CO_2, and C_1 compounds can be directly metabolized by methanogens to CH_4 and CO_2. Reducing equivalents are produced as a result of the fermentation, which must be removed so that electron carriers are re-oxidized, and therefore fermentation continues. One way is the reduction of pyruvate, mainly produced from sugars by fermenting bacteria via glycolysis, to lactate and ethanol. Pyruvate can also be oxidized to acetyl-CoA, further utilized by acetogenic bacteria (mostly *Firmicutes*) to form acetate by reducing CO_2 to CO with H_2, with the final incorporation of a methyl group [55]. Electrons can also be sinked into H_2, which is subsequently used by methanogens. Thus, methanogens play an important role catalyzing the last step of the anaerobic fermentation of organic polymers by influencing on the H_2 partial pressures, which influences the direction of

the fermentation [56]. Although bacteria are the main actors for primary fermentation, fungi and some protozoa may also perform this task [2].

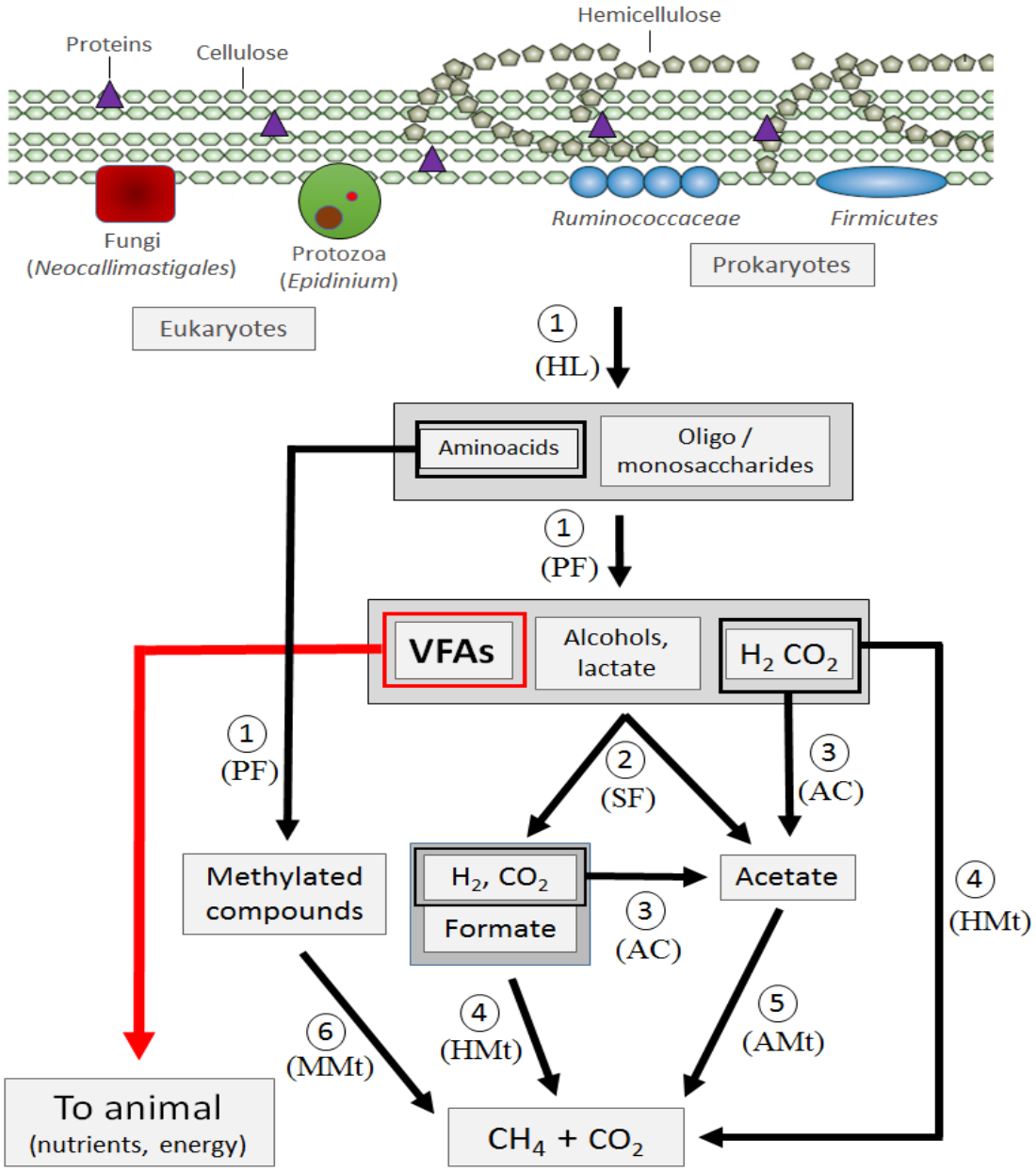

Fig 1. Schematic representation of the different steps and microbial groups during anaerobic catabolism of organic polymers. The substrates / byproducts driving or resulting from each metabolic reaction are in boxes. Double-boxing illustrates additional pathways for specific substrates. Substrates absorbed and used in the metabolism of the host are highlighted in red. The various microbial groups involved at each stage are numbered from 1 to 6: 1. hydrolytic and primary-fermenting bacteria; 2. secondary-fermenting bacteria; 3. acetogenic bacteria; 4. hydrogenotrophic methanogens; 5. aceticlastic methanogens; 6. methylotrophic methanogens. HL: hydrolysis of organic polymers; PF: primary fermentation; SF: secondary fermentation; AC: acetogenesis; HMt: hydrogenotrophic methanogenesis; AMt: acetotrophic methanogenesis; MMt: methylotrophic methanogenesis. Modified and combined from [2,54]. Reprinted with permission.

5.2.3. Use of VFAs in host metabolism

VFAs are saturated aliphatic organic acids consisting of one to six carbon atoms, presented in a straight or branched configuration. There are several type of VFAs, but acetate, propionate and butyrate are the most predominant forms in the rumen and hingut [57]. The VFAs produced by fermentative processess are largely absorbed (and metabolized) through the epithellial cells of the rumen (or hindgut), transported by the bloodstream, and finally incorporated in the metabolism of the host. In ruminants, VFAs are the major source of energy, constituting up to 70% of the total energy requirements [57]. For instance, propionate is the major substrate for gluconeogenesis in ruminants [58]. Hindgut fermentation also provides with a valuable amount of VFAs to ruminants, being the major source of such compounds in hindgut fermenters [57]. Acetate is usually present at a higher proportion than propionate or butyrate, but their respective proportions may greatly vary with diet. Diets rich in starch would promote propionate formation, whereas fiber digestion results in increased acetate production [59,60]. An imbalance in the ratio of these VFAs may influence the pH in the rumen or hindgut, which in some cases might be detrimental for the health of the host [61].

5.2.4. Amino acid fermentation

The fermentation of amino acids is a key process for the mineralization of proteins under anaerobic conditions. Proteins are usually degraded by the same microbes involved in carbohydrates fermentation through the synthesis and release of extracellular enzymes (proteases) cleaving the peptide bonds between amino acids (Fig. 2) [62]. Free amino acids are fermented into several products such as VFAs, ammonia, CO_2, H_2 and S-compounds, mainly through two major pathways: pairs of amino acids degraded simultaneously (Stickland reaction), or single-amino acid fermentation coupled with H_2-utilizing microbes, e.g. methanogens [63]. In the rumen, proteolytic bacteria are mostly associated to the genus *Prevotella* (*Bacteroidetes*), and *Butyrivibrio* (*Firmicutes*) [62]. Degradation of nitrogen-derived compounds (e.g. urea, amino acids) by the symbiotic gut microbiota may provide the host with a valuable source of nitrogen in situations with low protein availability [64].

5.2.5. Secondary fermentation

Secondary fermentation involves the reduction of substrates from primary fermentation such as alcohols and VFAs into smaller compounds, usually H_2, CO_2, formate and acetate (Fig. 2). These byproducts of fermentation are further utilized as metabolic substrates by several microbial groups, thus maintaining their levels low enough to preserve the exergonic character

of those reactions [54,65]. The β-oxidation of butyrate via crotonyl-CoA and 3-hydroxybutyryl-CoA into acetate, or the degradation of propionate through the reversible methylmalonyl CoA pathway into CO_2, acetate, and formate, are two examples of secondary syntrophic fermentation [66]. In both cases, the oxidation of reducing equivalents (e.g. NADH) is physically coupled to the production of H_2 further used by hydrogenotrophic microbes like methanogens [66]. Bacteria classified within the phyla *Firmicutes* and *Proteobacteria* are the main responsible for secondary fermentation.

5.3. CH_4-related processes

5.3.1. Methanogenesis and type of methanogens

Methanogenic archaea are strict anaerobic microorganisms, which are responsible for the production of CH_4 in habitats devoid of O_2 and other electron receptors [67]. Methanogenesis is a multi-step metabolic pathway mediated by a wide range of coenzymes used as electron donors (Fig. 3). The last steps of methanogenesis are common to all methanogens: first, a methyl group is transferred to CoM, forming methyl-CoM, which is subsequently reduced to CH_4 by a methyl coenzyme M reductase [67,68]. Enzymes and encoding genes mediating the different steps in CH_4 formation are characterized in detail (http://genome-jp/kegg/).

Phylogenetically, all belong to the archaeal phylum *Euryarchaeota*, and are traditionally divided into six orders, namely, *Methanomicrobiales, Methanobacteriales, Methanococcales, Methanosarcinales, Methanosaetaceae, Methanopyrales*; however, a new order (*Methanoplasmatales*) has been characterized recently [69]. Despite their phylogenetic diversity, methanogens are restricted to a few initial substrates: CO_2, methyl-group containing compounds, acetate, and marginally ethanol or propanol [68]. Most methanogens produce CH_4 by the hydrogenotrophic pathway involving the reduction of CO_2 to CH_4, and using H_2 as initial electron donor. Formate can also be used as electron source, but it requires to be previously oxidized to CO_2 by a formate dehydrogenase [67]. Microorganisms belonging to *Methanobacteriales*, *Methanomicrobioles, Methanococcales,* and some minor groups within *Methanosarcinales* follow this pathway. CH_4 production from acetate (acetoclastic) is restricted to methanogens within the genera *Methanosarcina* and *Methanosaeta* [67]. Methylotrophic methanogenesis, utilizing methyl-containing compounds (e.g. methanol, methylated amines, methylated sulfurs, etc.) as the initial substrate, is performed by *Methanosarcinales*, the newly described order *Methanoplasmatales*, and species within the genus *Methanosphaera* (order *Methanobacteriales*) [67,69].

5.3.2. CH_4 oxidation

Although mostly aerobically, microbial CH_4 oxidation in anoxic conditions also occurs [70]. Under anaerobiosis, CH_4 is oxidized to CO_2 and H_2 and it is suggested to be coupled to several reductive reactions such as sulphate, nitrate, iron, and manganese reduction [70]. Isolation of CH_4-utilizing bacteria from rumen samples indicates the existence of CH_4 oxidation, but it usually accounted for a residual fraction of the CH_4 produced [71].

5.3.3. Enteric CH_4 production

The CH_4 resulting from anaerobic catabolism is of no biological value for the animal host or used by the other syntrophic microorganism (apart from CH_4-oxidation). Thus, enteric CH_4 is disposed by the herbivore, mostly exhaled through mouth and nose, and it constitutes a loss of metabolic energy [72]. Total enteric CH_4 volume depends on several parameters, including the quality and the amount of feed ingested, the animal species, the composition of their symbiotic gut microbiota, and the density of methanogens. In domestic ruminants the percentage of gross energy (GE) intake, i.e. the total amount of chemical energy derived from the diet or the energy consumed, lost as CH_4 ranges 2-12% [73].

CH_4, together with CO_2 and nitrous oxide (N_2O), is one of the major greenhouse gases (GHG) contributing to Global Warming by its accumulation into the atmosphere [74]. CH_4 molecules possess 25 times higher Global Warming Potential, or capacity to trap heat in the atmosphere compared to CO_2, [75]. Approximately 20% of the Global Radiative Forcing (changes between input / output radiation) is attributed to atmospheric CH_4. As much as 22% of global man-related CH_4 emissions are estimated to result from enteric fermentation mostly from domesticated ruminants such as cattle, goat, camel, and buffalo, but also hindgut fermenters (e.g. horses) [75]. Avian-related CH_4 emissions are mainly calculated on records from poultry farms (hens and pullets) and they constitute a minor source of the total amounts; with few studies recording direct CH_4 emissions by birds [76,78]. In Norway, enteric fermentation contributed to 88% of the overall CH_4 emissions from agriculture and 4.2% of the national GHG emissions [78]. Reindeer has been calculated to release roughly 0.014 tonnes/animal/year of CH_4 [78].

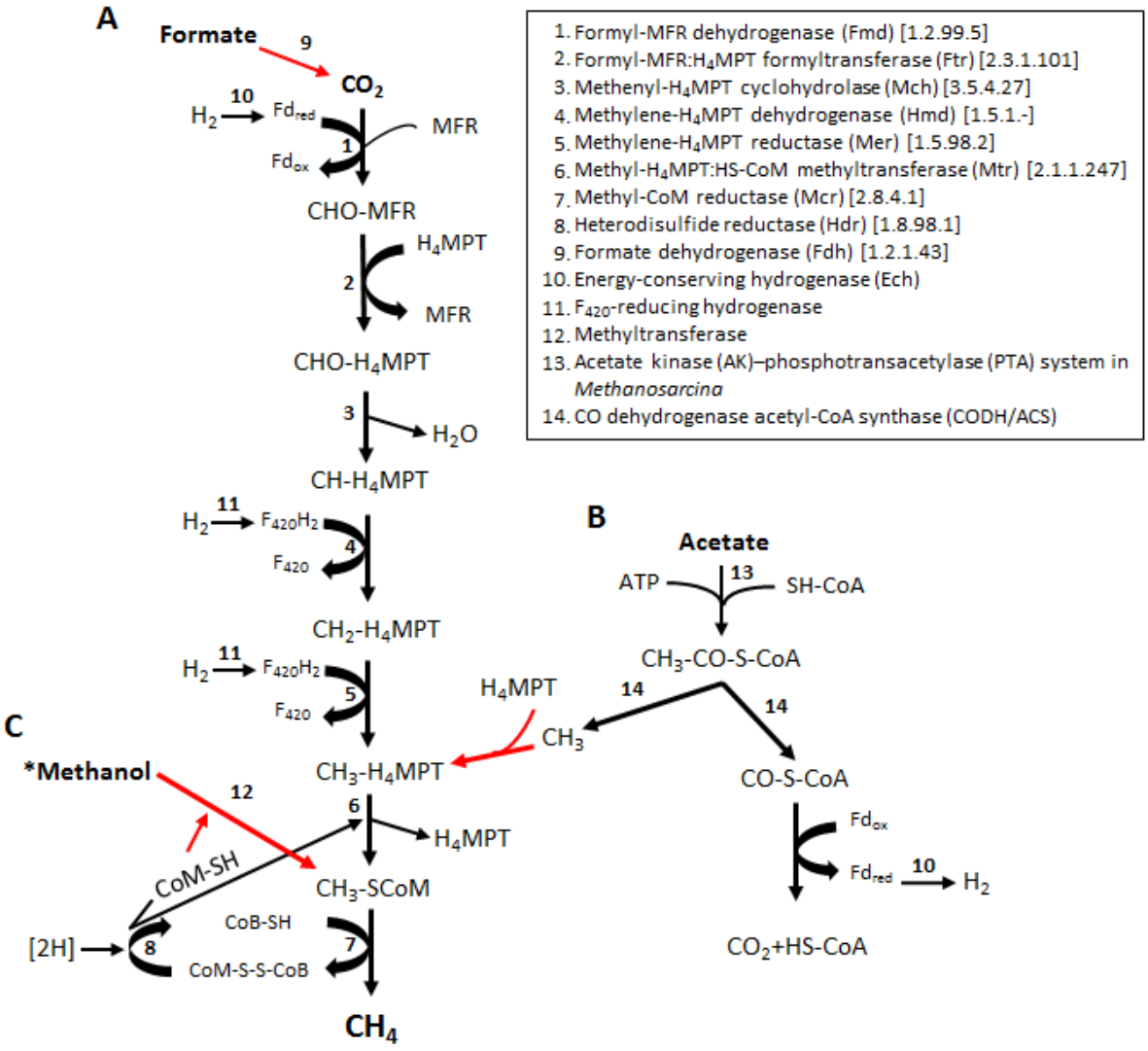

Fig 3. Schematic representation of the three main pathways of methanogenesis: **A)** hydrogenotrophic (also formate), **B)** aceticlastic, and **C)** methylotrophic methanogenesis. The full name and KEGG entries for the enzymes for each metabolic reaction (1-14) are given in the legend box. Arrows were colored in red for initial substrates alternative to the most common methanogenic pathway described in gut systems (hydrogenotrophic methanogenesis). Abbreviations: Fd_{red}: reduced ferredoxin; Fd_{ox}: oxidized ferredoxin; $F_{420}H_2$ reduced coenzyme F_{420} ; F_{420}: oxidized coenzyme F_{420} ; MFR: methanofuran; H_4MPT: tetrahydromethanopterin; CoM-SH: coenzyme M; CoB-SH: coenzyme B; CoM-S-S-CoB: heterodisulfide of CoM and CoB; SH-CoA: coenzyme A; CHO-MFR: formyl-MFR; CHO-H_2MPT: formyl-H_4MPT; CH-H_4MPT: methenyl-H_4MPT; CH_2-H_4MPT: methylene-H_4MPT; CH_3-H_4MPT: methyl-H_4MPT; CH_3-SCoM: methyl-CoM; CH_3-CO-S-CoA: methyl-AcylCoA; CO-S-CoA: acyl-CoA; CoA-SH: coenzyme A. * Electrons for the reduction of methyl groups are obtained by methyl-group oxidation to CO_2 reversible to methanogenesis. Modified from [67]. Reprinted with permission.

5.3.4. CH_4 mitigation strategies

Several strategies focused on a direct or indirect reduction of enteric CH_4 emissions from livestock have been devised [79]. Diet composition, i.e. type and proportion of some specific polysaccharides, may substantially influence anaerobic fermentation and CH_4 production. Dietary starch and easily fermentable (short-chained) carbohydrates promote increased

fermentation and total VFAs production, especially propionate [60]. Such increase in VFAs causes a drop in ruminal pH that may affect the gut microbiota, in some cases leading to depressed methanogenesis [60]. Grinding of the food before ingestion also reduces methanogenesis by increasing the passage of food through the gut system and limiting the time available for CH_4 production [79]. Some organic acids (malate, fumarate) may also lead to reduced methanogenesis by acting as alternative H_2-sinks, thus diverting H_2 from CH_4 metabolism [79].

5.4. Plant Secondary Metabolites (PSMs)

5.4.1. Role of PSMs in plants and effects on herbivores

An alternative strategy for the abatement of CH_4 is the use of diets high in PSMs or specific PSMs as dietary supplements [79]. PSMs are constituted by a vast array of non-nutritive organic compounds (~200,000 identified), categorized into several groups based on their chemistry and structure: saponins, tannins, flavonoids, essential oils (EO) and organosulphur compounds (OS) [80]. These compounds are not directly involved in primary biochemical processes such as reproduction, growth or development [81,80]. Instead, PSMs play an important role as part of the plant chemical defenses: protection against herbivores, pests and microbes [80,82]; or reflection of UV radiation from the sun [83], among others. Their bitter taste may also deter herbivores from ingesting the plant (deterrent effect) [56]. Some PSMs may also directly bind to proteins when ingested, forming complexes that cannot be dissociated by the enzymes produced by the host and finally not digested [84]. This would result in an impoverished nutritional status and consequently less investment in reproduction and offspring nutrition decreasing reproductive success [85]. A cytotoxic and tissue lytic effect have been described to several PSMs, leading to organ damage like in the liver, kidney or skeletal muscle [86]. In some extreme cases may lead to death [87].

5.4.2. PSMs detoxification strategies

Mammalian herbivores consuming foodstuffs high in PSMs have developed endogenous strategies driving the direct detoxification of such compounds. PSMs detoxification usually involves two phases: 1) functionalization and 2) conjugation (Fig. 4). Functionalization involves the addition of various functional groups that cause the oxidation, reduction or hydrolysis of the secondary compounds. Chemical conjugation consists in the addition of water-soluble conjugates (e.g. a sugar, sulphate group or amino acid moiety) so that the compound becomes more hydrophilic, being finally excreted by urine or bile [88,89]. Plant toxins may also bind directly to the epithelial cells lining the gut being subsequently transported out to the gut

lumen by efflux transporters [90]. In addition, some herbivores also produce tannin-binding salivary proteins forming stable tannin-protein complexes finally excreted in the feces [91].

5.4.3. Anaerobic degradation of PSMs

Aromatic compounds as those constituting the core of many PSMs are degraded under anoxic conditions through a series of reductive reactions performed by the symbiotic microbiota [92]. Bacterial degradation of aromatics first involves the use of a wide repertoire of peripheral metabolic pathways specific to the chemical nature for each family of compounds (Fig. 5). These reactions result in a more limited range of common intermediates further metabolized through a few central pathways. The aromatic ring of these intermediates is reduced (dearomatization), and dearomatized products are further metabolized following common pathways for carbon metabolism in the form of, e.g. VFAs, acetyl-CoA, pyruvate, etc (Fig. 5) [92].

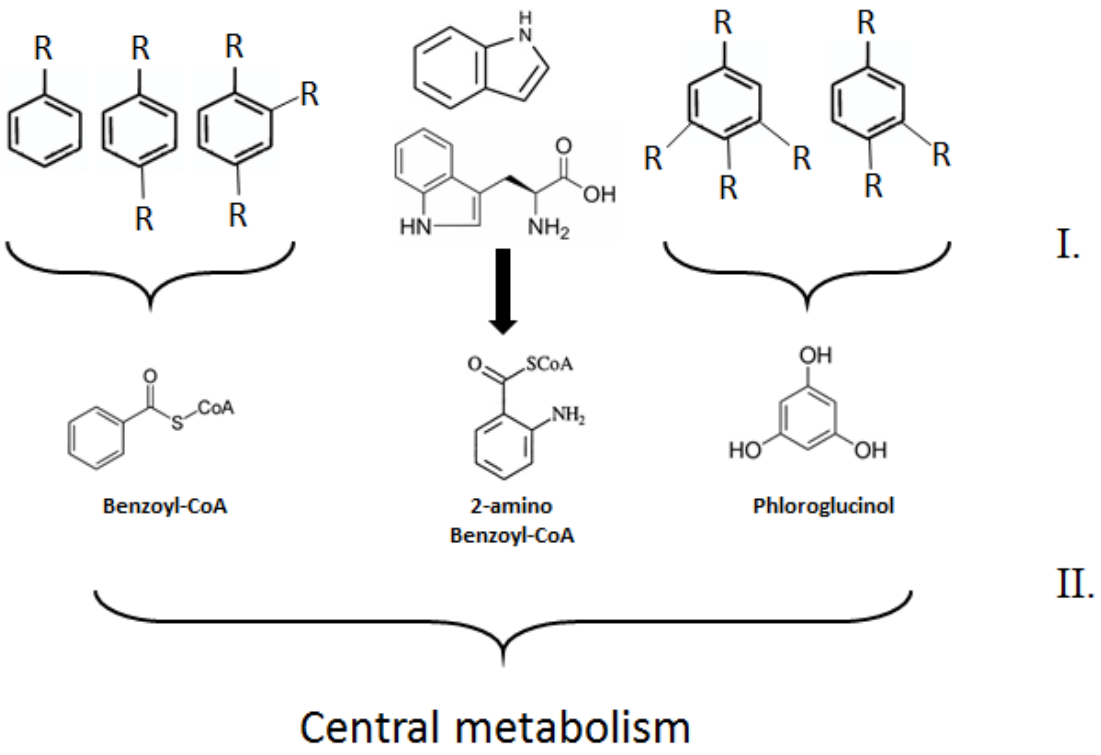

Fig 4. Mechanisms involved in the anaerobic biodegradation of aromatic compounds. Anaerobic microbial biodegradation generally consists of two major processes: (I) a wide range of aromatic compounds are processed through specific peripheral pathways into a few key components (II) whose aromatic rings are further reduced (dearomatization). The resulting byproducts are finally used in the central metabolism of the cell. Based on [92].

As in other catabolic reactions under anaerobiosis, fermentation of aromatic compounds demands the co-participation of other microbial groups (e.g. hydrogenotrophic methanogens) in order to remove metabolic end products like H_2 [93]. Coupling with anaerobic respiration

may also occur with electron acceptors such as nitrate, sulfate, iron(III), etc. The enzymes and metabolic pathways involved in every step of the anaerobic catabolism of these compounds have been described in detail (http://www.genome.jp/kegg/). *Proteobacteria* is the major phylum for aromatics-degrading bacteria, but some bacteria within the genus *Eubacterium* were also reported to degrade aromatic compounds [92,94].

5.4.4. Effects of PSMs on methanogenesis

The strong microcidal and microstatic properties reported to several PSMs may alter the growth of the different microbes dwelling the digestive tract of herbivores, potentially affecting anaerobic fermentation and methanogenesis [56]. Fig. 5 illustrates the different mode of actions whereby phytochemicals may alter methanogenesis [80]. 1) PSMs may negatively affect methanogenesis by the direct inhibition of methanogens; 2) indirectly through an inhibition of methanogens-associated protozoa, leading to the accumulation of H_2 that may hamper fiber degradation; 3) through the direct inhibition of cellulolytic bacteria and fungi causing a reduction in fiber digestion, and therefore methanogenesis; 4) anaerobic metabolism can also be redirected towards pathways resulting in less CH_4 yields (e.g. propionate over acetate production) [80]. The production of propionate may act as an alternative H_2-sink metabolic pathway to the formation of CH_4 [79]. However, the effect on the metabolism may greatly vary depending on the type of PSMs [80]. Total VFAs production can also be affected by PSMs although parameters such as dose, experimental conditions or dietary composition may lead to variable outcomes [56,95].

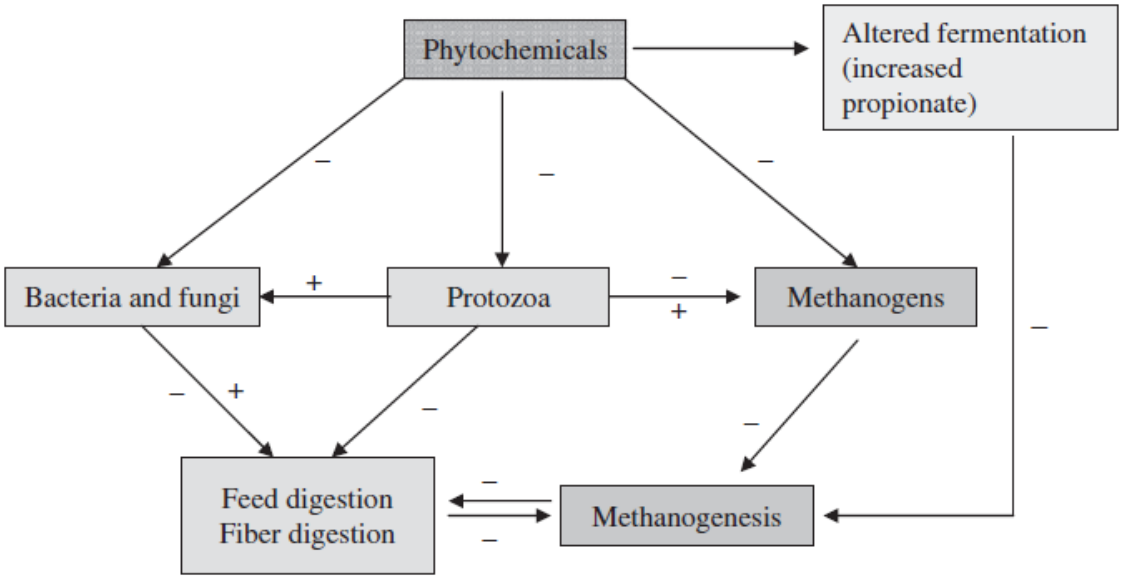

Fig 5. Diagram representing a proposed mode of action whereby PSMs may affect methanogenesis. Either the effect on the different constituents involved in anaerobic degradation is positive (+) or negative (-) depends on the type and dose of the phytochemical, and diet composition. From [80]. Reprinted with permission.

6. Animals investigated

6.1. The Arctic region

All the animals included in this thesis shared, apart from being herbivores, the characteristic of inhabiting Arctic and sub-Arctic environments. The Arctic is the region located at the northernmost part of the Earth, geographically defined as the area above the Arctic Circle (66° 33'N) where complete darkness (polar night) in winter and constant light (midnight sun) in summer occurs. From an ecological standpoint, the Arctic is the area whose average temperature for the warmest month (usually July) remains below 10 °C [96], but these temperatures widely vary among the Arctic regions throughout the year. Europe and Western Scandinavia possess a moderate climate produced as a direct consequence of the North Atlantic Drift Current (NADC), a unique water current derived from the Gulf Stream-North Atlantic Current [97]. It involves a slow, wind-driven flow of warm water covering the eastern subpolar North Atlantic. Vegetation is severely restricted by climatic conditions (mainly water availability) to a few months during summer in the northernmost Arctic areas. Grasses, sedges, willow shrubs, dwarf birch, and lichens (mainly eaten by reindeer and muskoxen) mostly constitute the "Arctic flora", with distribution and diversity strongly influenced by climate [98]. UV-B radiation is predicted to increase at high latitudes thus increasing the concentration of some PSMs (e.g. usnic acid in lichens) as they are partly produced as a reponse to sun radiation due to their screening properties [83]. Increasing dietary PSMs contents might lead to changes in the gut microbiota of herbivores feeding on these foodstuffs towards the presence of microbial groups involved in PSMs degradation in order to cope with their potential toxicity.

6.2. Norwegian reindeer (*Rangifer tarandus tarandus*)

Reindeer (Fig. 6a) are ruminants within the order *Artiodactyla*, or even-toed ungulates, belonging to the *Cervidae* family. Reindeer currently accounts for approximately 5 million animals divided in seven sub-species with a circumpolar distribution around the Northern hemisphere [99].

The subspecies Eurasian tundra or Nowegian reindeer constitutes around two million animals herded on natural pastures by 24 indigenous groups, mostly in the regions of Fennoscandia (Northern Norway, Sweden and Finland) and northern Russia [100]. In Norway, there are approximately 250,000 semi-domesticated reindeer herded by the Sami people and two non-Sami herds [101], in addition to around 25,000 wild reindeer. Norwegian reindeer are migratory animals, displaying seasonal shifts from coastal areas with lush vegetation in summer towards inland lichen-range in winter. Vascular plants like herbs, shrubs, graminoids,

lichens and mosses are included in their diet; however, their relative proportions will vary with the season [102,103]. Based on the anatomy of their feeding apparatus, with narrow, pointed muzzles, and their digestive system Norwegian reindeer are classified as intermediate feeders better adapted to the ingestion of high-quality forages as those found in summer [103].

Lichens are symbionts of algae and fungi, rich in energy, that make them an important food item for Norwegian reindeer during winter [104]. Lichens are almost devoid of proteins, making essential for Norwegian reindeer to include nitrogen-rich vascular plants in order to meet their nutritional demands in winter [105]. The chemical composition of lichens differs from vascular plants and shows a high structural variability among species [105]. Lichens are also rich in PSMs [106], but they can be fully degraded by the rumen microbiota in these ruminants [107]. Noticeably, the consumption of a pure lichen diet resulted in lower CH_4 emissions compared to Norwegian reindeer fed pellets concentrate [108]. The rumen microbiota in these ruminants is mostly constituted of bacteria from the phyla *Firmicutes* and *Bacteroidetes*; archaea from the genus *Methanobrevibacter*; protozoa from the genera *Entodinium*, *Isotrichia* and *Epidinium*; and fungi assigned to the order *Neocallimastigales* [3,11,12]. PSM-tolerant bacterial strains were isolated from their rumen contents, which may explain the high tolerance to lichens displayed by reindeer [109,110].

Fig 6. Animal subjects whose gut samples were used in this thesis. (A) Norwegian reindeer (photo: Monica A Sundset). (B) Muskoxen (photo: Lorenzo Ragazzi). (C) Svalbard rock ptarmigan (photo: Nicolas Lecomte). All photos are printed with permission.

6.3. Muskox (*Ovibos moschatus*)

Muskoxen are large arctic ruminants belonging to the order *Artiodactyla* but phylogenetically related to members of the family *Bovidae*, such as goat and sheep. (Fig. 6b) Ancient DNA analyses indicated that muskoxen possessed a Holarctic distribution, being finally extinct on their Eurasian range at the beginning of the Holocene [111]. Current natural populations of muskoxen are mostly found in northern Canada and some regions in the north- and north-east of Greenland. Some small populations have been established into north and west Alaska, and

in some areas of Russia, Canada and south and north of Norway [111,112]. The current population of muskoxen is approximately 125,000 animals worldwide (https://www.fws.gov/).

The pelage of muskoxen is constituted by two layers of fur that allows them to survive under extremely low temperatures common in such habitats. Very long and dark hairs constitute the outer layer of fur, coating the inner and softer wool (Inukitut: Qiviut), with exceptional insulating properties [113]. The anatomy of their digestive tract consist of a large rumen-reticulum, a large omasum and a relatively small caeca-colon, contributing to extremely long food retention times [114,115]. Despite their bulky appearance, muskoxen possess narrow muzzles and low crowned molariform teeth specific for selective feeders / browsers [103]. They mainly graze on highly lignocellulosic forage all-year-round excepting in the short, lush arctic summer, during which they ingest highly nutritious food [116,117]. Lichens and willow (*Salix* spp.) are included during summertime, and chemical analysis of the latter has reported a high proportion of PSMs such as flavonoids and phenolic compounds [116,118]. The description of the eukaryotic metagenome reported a high proportion of putative GHs in the rumen of muskoxen, indicating the existence of a novel rumen microflora adapted to an Arctic diet [33]. No description of the prokaryotic gut microbiota from muskoxen has been attempted. Net CH_4 estimates for muskoxen fed brome hay are between 2.0-3.2% GE [119].

6.4. Rock ptarmigan (*Lagopus muta*)

Ptarmigans (Fig. 6c) are gallinaceous birds pertaining to the subfamily *Tetraoninae*, or the grouse family. In general, ptarmigans are non-migratory birds mostly found at arctic and sub-arctic latitudes, on rocky mountain sides, tundra, and some isolated populations in mountains of Scotland, the Alps, the Pyrenees, and the Urals in Europe as well central and eastern Asia [96]. There is no estimation on the population of rock ptarmigans worldwide. The broad geographical distribution displayed by the rock ptarmigan has led to different subspecies with diverse physical and phenotypical traits. The Svalbard rock ptarmigan (*L. m. hyperborea*), the only terrestrial bird to reside year-round in the Archipelago of Svalbard, possess a body size (53-40 cm) and weight (490-1200 g) generally higher than rock or willow ptarmigan from northern Norway. In addition, Svalbard ptarmigans show substantial annual body weight variations, low in summer and high during fall / Winter, achieved through augmented fat deposition exceeding 30% of total body weight [120]. Fat is accumulated as a preparation to the 4-months dark winter period when feeding is restricted because of icing, and in which reducing body heat losses are crucial for survival. Also feeding behavior decreases through winter to reduce energy expenditure from locomotion, facilitating fat deposition [120].

Diet composition greatly depends on the season: *Polygonum viviparum* is typically ingested in summer (some subspecies including also insects) due to their high protein contents, whereas the rest of the year it results in a mix of mainly *Salix* spp. and *Saxifraga* spp. [121]. Willow (*Salix* spp.) is of high nutritional value for ptarmigans but also possess a wide array of PSMs [118,122]. As other herbivorous birds, ptarmigans possess large paried fermentation chambers (caeca) protruding out from the ileo-colonic junction [123], and housing high concentrations of symbiotic microbiota involved in the fermentation of the plant material ingested by these birds [18,124]. These also mediate the metabolism of non-protein nitrogen (e.g. uric acid to ammonia or amino acids) resulting from the retrograde peristalsis of urine combined with feces, which may constitute an alternative source of nitrogen for the bird [64,125]. Ushida et al. [18] reported the presence of several microbial groups in the ceca of Japanese rock ptarmigans, mostly belonging to he phyla *Actinobacteria, Firmicutes, Bacteroidetes* and *Proteobacteria* [18]. CH_4 records from ptarmigans are limited to only one study using captive rock ptarmigans, releasing proportionally lower CH_4 than for ruminants [76].

7. Methods

Approximately 2% of the total bacteria are estimated to be cultivable in the laboratory, a fact that leaves a sheer amount of microbes to be characterized. The inherent bias of culture-based techniques has been circumvented with the advent of molecular biology, which allows the study and characterization of mixed microbial communities. Massive parallel amplicon sequencing methods not only allows obtaining information of the dominant microorganisms in a specific environment, but also give information of low-abundance microbes comprising the so-called 'rare' microbiota [126]. Despite being extensively used for community characterization, there exist several flaws inherent to 16S rRNA-based sequencing techniques: relatively short read lengths; the occurrence of sequencing errors; primer pairs targeting different regions; or unreliable Operational Taxonomic Unit (OTU) classification [127]. All these issues may increase uncertainty on diversity estimations and on the taxonomical classification.

Microbiota-related genes (i.e. the metagenome) extensively outnumber the genetic material from the host [128]. Shotgun metagenome sequencing has been widely used to obtain an accurate picture of this metagenome. In brief, it allows the sequencing of all genes present in a specific environment without the need of previous amplification steps that may add some potential bias. Genetic information is then aligned and compared with different genomic databases (RefSeq, non-redundant NCBI, Pfam) [129], and finally annotated to functional groups for its biological interpretation using curated databases (KEGG, KO, SEED) [130]. As a

result, an improved interpretation of the genetic material related to this microbiota is achieved, which gives the possibility to characterize the main contributors to the different metabolic steps in the anaerobic catabolism of polymers.

In this thesis, two main sequencing techniques: **ROCHE 454** pyrosequencing (amplicon sequencing for 16S rRNA taxonomy) and **Illumina HiSeq** (shotgun metagenomics) were applied. Both platforms presented different specifications that made them suitable for their respective goals. For instance, the longer reads obtained with 454 pyrosequencing allows taxonomical classification at a deeper level, whereas Illumina yields a higher amount of sequences (millions of sequences per sample versus thousand for 454) thus being suitable to cover the sheer number of genes found in a single sample.

One inherent limitation to both sequencing techniques is that they only provide with a relative estimation for the different microbial groups identified in a sample. Accordingly, in this thesis, a quantitative real-time PCR (qRT-PCR) technique was also used to accurately calculate the concentration of the main microbial constituents (bacteria, archaea and protozoa).

8. Summary of papers

PAPER I: Rumen and cecum microbiomes in reindeer (*Rangifer tarandus tarandus*) are changed in response to a lichen diet and may affect enteric methane emissions

Previous experiments conducted in the Department of Arctic and Marine Biology at UiT – The Arctic University of Tromsø, described lower methane output from Norwegian reindeer fed solely on lichens compared to an "artificial" pelleted feed. The high content of PSMs was suggested as one the major accounts for reduced CH_4 yields with animal feeding on lichens, although dissimilar carbohydrate contents between diets was also considered. In **PAPER I** we identified and compared the rumen and cecum microbiota between both groups of Norwegian reindeer fed either lichens or grass-based pellets. Differences between their respective microbial communities, especially methanogens, may partly account for the observed reduction in CH_4 emission with the ingestion of a lichen-based diet. qRT-PCR was used to calculate concentration of methanogens, bacteria, and protozoa. Taxonomy was obtained applying amplicon 16S rRNA gene sequencing and analysis (archaea and bacteria).

Main results:

- No differences in the concentration of rumen methanogens between both groups of Norwegian reindeer.

- Archaeal and bacterial communities in the rumen and cecum were significantly different between Norwegian reindeer fed lichens or pellets.
- *Methanobrevibacter thaueri* was the major archaeal phylotype found with both diets.
- The relative proportion of SGMT-methanogens (*Methanobrevibacter smithii, Methanobrevibacter gottschalkii, Methanobrevibacter millerae, and Methanobrevibacter thaueri*) over RO-methanogens (*Methanobrevibacter ruminantium, Methanobrevibacter olleyae*) was reduced due to an increase in the latter group in Norwegian reindeer fed lichens.
- A lower relative proportion of predicted genes involved in CH_4 metabolism was observed in lichen-fed Norwegian reindeer.
- A trend of increasing *Firmicutes* bacteria over *Bacteroidetes* was observed with the ingestion of lichens.

Main conclusions:

- The ingestion of lichens led to different archaeal and bacterial profiles.
- A trend of decreased ratio between SGMT-methanogens and RO-methanogens with the ingestion of lichens may suggest increased relative proportion of RO-methanogens associated to lower CH_4 output, as discussed in other ruminants (e.g. cattle).
- Lower abundance of predicted genes associated to CH_4 metabolism with the ingestion of lichens supports previous findings associating this diet with reduced methanogenesis in Norwegian reindeer.

PAPER II: <u>First insight into the faecal microbiota of the high Arctic muskox (*Ovibos moschatus*)</u>

Muskoxen are large ruminants well-adapted to the Arctic environment, feeding typically on forages high in lignocellulose excepting in the short arctic summer. There is only one study describing the rumen eukaryotic microbiota in muskoxen but not on the prokaryotic fraction. One previous calculation on CH_4 records from muskoxen (fed graminoids / brome hay, high in PSMs) indicated around 2.0-3.2% of GE intake lost as CH_4, lower than in domesticated ruminants. It may indicate a specific microbiota linked to low CH_4 yields in the muskoxen. In **PAPER II** we characterized, for the first time, the prokaryotic fecal microbiota from a group of semi-domesticated muskoxen located in northern Norway, in September. More knowledge on this microbial consortium would allow a better understanding on the digestive physiology in these ruminants. Fecal droppings were used as a proxy to obtain a first picture of this microbial

consortium. Amplicon 16S rRNA sequencing was applied to identify the major archaeal and bacterial community profiles.

Main results:

- *Firmicutes* and *Bacteroidetes* were the major bacterial phyla, with genera associated to the family *Ruminococcaceae* as the dominant bacterial group.
- More than 50% of total bacterial sequences were assigned to 'uncharacterized' bacterial phylotypes.
- Archaeal community was mostly dominated by *Methanobervibacter* spp., followed by *Methanomassiliicoccaceae* methanogens.
- Comparisons with other ruminant and non-ruminant herbivores inhabiting lower latitudes indicated that archaeal and bacteria profiles in muskoxen are unique but more similar to other Arctic ruminants like Norwegian reindeer.

Main conclusions:

- Dominance of putative fibrolytic bacteria (e.g. some *Ruminococcus* spp.) agrees with a typical fiber-rich diet for muskoxen in fall/winter.
- Large abundance of 'uncharacterized' bacteria suggests the presence of novel bacterial taxa adapted to the deconstruction of polysaccharides from Arctic plants. This result is also supported by comparisons between the muskoxen microbiome and datasets from other herbivores at lower latitudes.
- Description of fecal methanogens may be useful as a first approximation on CH_4 microbiology in muskoxen but information on rumen archaea as well as direct CH_4 measurements are essential to better understand methanogenesis in muskoxen.

PAPER III: <u>Characterization of the cecum microbiome from wild and captive rock ptarmigans from Svalbard and northern Norway in relation to diet composition</u>

Rock ptarmigans are gallinaceous birds with a changeable diet according to the season, but usually constituted by plants (or parts of plants) of high-nutritional value that may also possess high PSMs contents. One previous study described the cecal microbiota from wild Japanese rock ptarmigans but no information on wild rock ptarmigans from Svalbard and Scandinavia has been attempted. Avian-related methanogenesis has been neglected compared to that from ruminants, and CH_4 records from captive rock ptarmigans estimated 0.2-0.4% of metabolic energy (ME) lost as CH_4. In **PAPER III** we investigated, for the first time, the cecal microbiome (microbial taxonomy and related gene contents) in wild rock ptarmigans from

Svalbard and northern Norway. Characterizing this microbiome would help better understand the digestive physiology in these birds and the potential link between diets high in PSMs, microbial taxonomy, and related metabolic processes (e.g. methanogenesis). The cecal microbiome from captive Svalbard rock ptarmigans fed a commercial diet was also described to investigate the effects of diet composition on all these parameters. Amplicon 16S rRNA sequencing was applied to identify the major archaeal and bacterial taxa, and whole gene contents from this microbiota were investigated using shotgun metagenomics. qRT-PCR was used to quantify bacteria and methanogens.

Main results:

- Concentration of methanogens and bacteria were higher in wild ptarmigans compared to captive ptarmigans.
- *Methanomassiliicoccaceae* was the major archaeal family found in wild ptarmigans followed by *Methanocorpusculaceae*. *Methanobrevibacter* spp. dominated in captive ptarmigans but generally archaeal contents were low.
- Higher bacterial diversity in wild ptarmigans: Families A*ctinobacteria, Firmicutes, Bacteroidetes, Synergistetes, Spirochaetes* were present; *Firmicutes* was the major phyla in the captive groups followed by *Bacteroidetes* (at a minor proportion).
- CH_4-related genes were found at a higher abundance in wild ptarmigans with genes involved in methanogenesis from several substrates (e.g. H_2, methanol, formate, etc).
- Genes mediating the degradation of xenobiotics were present at higher relative abundances in wild ptarmigans. Genes for a pathway involving degradation of phenol/catechol into pyruvate were only found in wild birds.
- High richness of hydrolytic enzymes in wild and captive ptarmigans, mostly involved in the degradation of hemicellulose, starch, and pectin.

Main conclusions:

- Wild ptarmigans possess a diverse cecum microbiota including several bacterial groups involved in the degradation of PSMs (e.g. *Synergistetes, Coriobacteriaceae*).
- Methanol-fueled methanogens associated to *Methanomassiliicoccaceae* were dominant presumably due to methanol production resulting from the depolymerization of pectic compounds.
- A higher CH_4 potential was observed in wild ptarmigans, with higher concentrations of methanogens and relative abundances of genes mediating methanogenesis. A natural diet with a broader range of polysaccharides as those ingested by wild ptarmigans

would lead to longer fermentation times allowing higher microbial growth leading to such differences.

- High relative abundances of enzymes involved in the hydrolysis of hemicellulose indicates a diet with high fiber contents, at least in fall/winter. Nonetheless, enzymes mediating the degradation of starch, pectin, may also reflect the capacity to select those parts of higher nutritional value from fibrous plants. These findings suggest a versatile cecum microbiota in ptarmigans capable to degrade foodstuffs with variable fiber contents that may be beneficial in periods scarce of high-quality food.

9. General discussion

9.1. Gut microbiota and methanogenesis

9.1.1. Archaeal profiles in relation to CH_4 emissions

In this PhD thesis, it we intended to relate the presence of specific methanogens with CH_4 potential from Norwegian reindeer, muskoxen and ptarmigan. CH_4 yields described for muskoxen and Norwegian reindeer were low compared to those reported to cattle, and emissions from lichen-fed Norwegian reindeer were lower than those fed a pelleted grass-based diet (Table 3) [108,119]. Overall, the archaeal profiles from Norwegian reindeer fed lichens were significantly different to those fed pellets concentrates (**PAPER I**), but the relative abundance of none of the major methanogens differed significantly. The differences became more apparent when methanogens were classified in two major clades, namely, SGMT-methanogens and RO-methanogens as described by King et al. [19] (Fig. 7). As indicated, the relative abundance of RO-methanogens increased in Norwegian reindeer fed lichens leading to a decrease in the value for the ratio SGMT:RO-methanogens (Rumen: Pellets = 12.1; Lichens = 1.9; Cecum: Pd = 12.4; Ld = 3.8) (Fig. 7). Noticeably, when we classified the methanogens found in muskoxen feces following the same classification system as we did with the data from Norwegian reindeer (Fig. 7), RO-methanogens constituted 42% of total sequences, and the SGMT:RO-methanogens ratio (SGMT:RO = 1) was close to those described in Norwegian reindeer consuming a lichen-based diet (rumen: SGMT:RO = 1.9; cecum: SGMT:RO = 3.8) (Fig. 7). The only report on CH_4 records from muskoxen grazing a fibrous diet indicated a low percent of GE intake lost as CH_4 (2.0-3.2%) [119], even lower than those from Norwegian reindeer fed lichens (5.1%) (Table 3) [108]. Considering that the dominance of SGMT-methanogens over RO-methanogens has been discussed to be positively correlated to higher CH_4 production in cows [131], we speculate that the opposite trend, i.e. an increase in the

relative abundance of RO-methanogens (leading to lower SGMT:RO ratio) may be linked to the low CH_4 yields reported to Norwegian reindeer fed lichens and muskoxen. In addition, CH_4 estimations from another arctic ruminant like the moose (*Alces alces*) accounted for only 2.1% loss of the GE intake lost as CH_4 when fed a mixed browse diet, rather similar to the CH_4 yields from muskoxen and lower than Norwegian reindeer fed lichens [132, see discussion (133)]. Characterization of the rumen methanogens from wild moose at different geographical locations showed most of the individuals housing a high proportion of RO-methanogens (resulting in low SGMT:RO ratios) [5]. Overall, these findings may suggest a putative link between increased relative abundance of RO-methanogens and low methanogenesis in muskoxen and Norwegian reindeer. Although this approximation may be valid for the Norwegian reindeer study, there exist some limitations to relate the archaeal profiles with low CH_4 output in muskoxen as only fecal samples were used in our study, and most of the enteric CH_4 is produced in their large reticulo-rumen (90%) [134]. Considering the small differences in the archaeal profiles obtained in the rumen and cecum of Norwegian reindeer fed pellets or lichens (**PAPER I**), we considered that using fecal samples may be a valuable first approximation to understand the ecology of methanogens in muskoxen. It would be necessary to characterize the rumen archaeal microbiota in addition to new CH_4 measurements in order to verify the relationship between a high relative abundance of RO-methanogens and low CH_4 output in muskoxen.

Table 3. Percentage of GE intake lost as CH_4 in different herbivores and their respective diets.

Animal source	**Diet**	**% GE intake lost as CH_4**	**Ref.**
Reindeer	Pelleted feed Lichens	7.6 ± 0.7 5.1 ± 0.9	[108]
Muskoxen	Brome hay	2.0-3.2	[119]
Moose	Mixed browse	2.1	[132,133]
Sheep	Pasture Lucerne *Lotus pedunculatus* (contains condensed tannins)	6.2 5.7 3.9	[135]
Cow	Ryegrass silage *Lotus corniculatus* (contains condensed tannins)	10.8 8.6	[135]
Rock ptarmigan	Pelleted feed + powdered cellulose	0.2-0.4*	[76]

*Data only referred as percentage of the metabolizable energy (ME), i.e. portion of the gross energy useful for metabolism. Percentage of GE intake lost as CH_4 was not available.

Methanomassiliicoccaceae was the dominant archaeal group in wild rock ptarmigans (**PAPER III**). Unfortunately, no CH_4 measurements were recorded from wild ptarmigans, thus making impossible to relate dominance by *Methanomassiliicoccaceae* with CH_4 emissions. However, previous CH_4 records from captive rock ptarmigans reported only 0.2-0.4% of the metabolic energy (ME) obtained from food lost as CH_4, much lower than in cattle (13.5-17%), sheep (3.9-6.2) or reindeer fed lichens (referred as GE intake lost as CH_4: 5.1%) (Table 3) [76,108,119,135]. When CH_4 losses were related to the total amount of energy derived from fermentation, the values from ptarmigans (12% of the GE intake lost as CH_4) were still lower than in ruminants (19%) [see discussion (76)]. No CH_4 records were detected from captive ptarmigans fed pellets concentrate at our department (unpublished data), supporting the low CH_4 yields from ptarmigans obtained in early studies. Our metagenomics study showed a higher concentration of methanogens and genes related to methanogenesis in wild ptarmigans compared to the captive group (**PAPER III**), which may indicate a higher CH_4 potential, i.e. higher methanogenesis, for this group. New analysis showed be focused on recording CH_4 emissions from wild ptarmigans, or birds fed a natural diet (not pelleted), to find out whether these birds also possessed relatively low CH_4 output and its relationship with dominance by *Methanomassiliicoccaceae*.

9.1.2. Effects of PSMs on archaeal profiles

PSMs and PSMs-rich diets have been widely reported to negatively influence some methanogens and methanogenesis [56,80]. Nonetheless, several factors should also be considered when assessing the potential effects derived from the ingestion of PSM-rich diets on the archaeal profiles. For instance, characterization of the rumen methanogens in Chinese sika deer (*Cervus nippon*) consuming an oak-leave diet (high in PSMs) also showed a lower relative abundance of SGMT-methanogens compared to a corn-based diet (low in PSMs), but in this case RO-methanogens were completely inhibited [23]. A similar study with the same animal fed diets with high or low tannin contents showed the opposite, i.e. no differences between groups and with RO-methanogens constituting a meaningful fraction of the archaeal microbiota [136]. These results suggest that inter-indivual variations may also influence on the potential effects resulting from the intake of diets high in PSMs on the archaeal taxonomy. Another factor to be considered is the role played by the gut microbiota in the degradation of PSMs, as reported in Norwegian reindeer [107]. The degradation of such compounds may bypass any potential effect on the archeal taxonomy, as it was discussed for the lack of differences observed in the concentration of methanogens between Norwegian reindeer fed lichens and pellets concentrates (Table 3-4, **PAPER I**).

Muskoxen naturally ingest PSM-rich plants (e.g. willow species) as part of their natural diets, especially during summer [116], but the fecal samples from the muskoxen used in our study were collected in September when these ruminants mostly graze on graminoids, and therefore may not include as much PSMs-rich foodstuffs (see methods **PAPER II**). The fact that muskoxen also presented a high proportion of RO-methanogens indicates the limitations of attempting to establish a link between the presence of a specific type of methanogens as a direct result of PSMs contents.

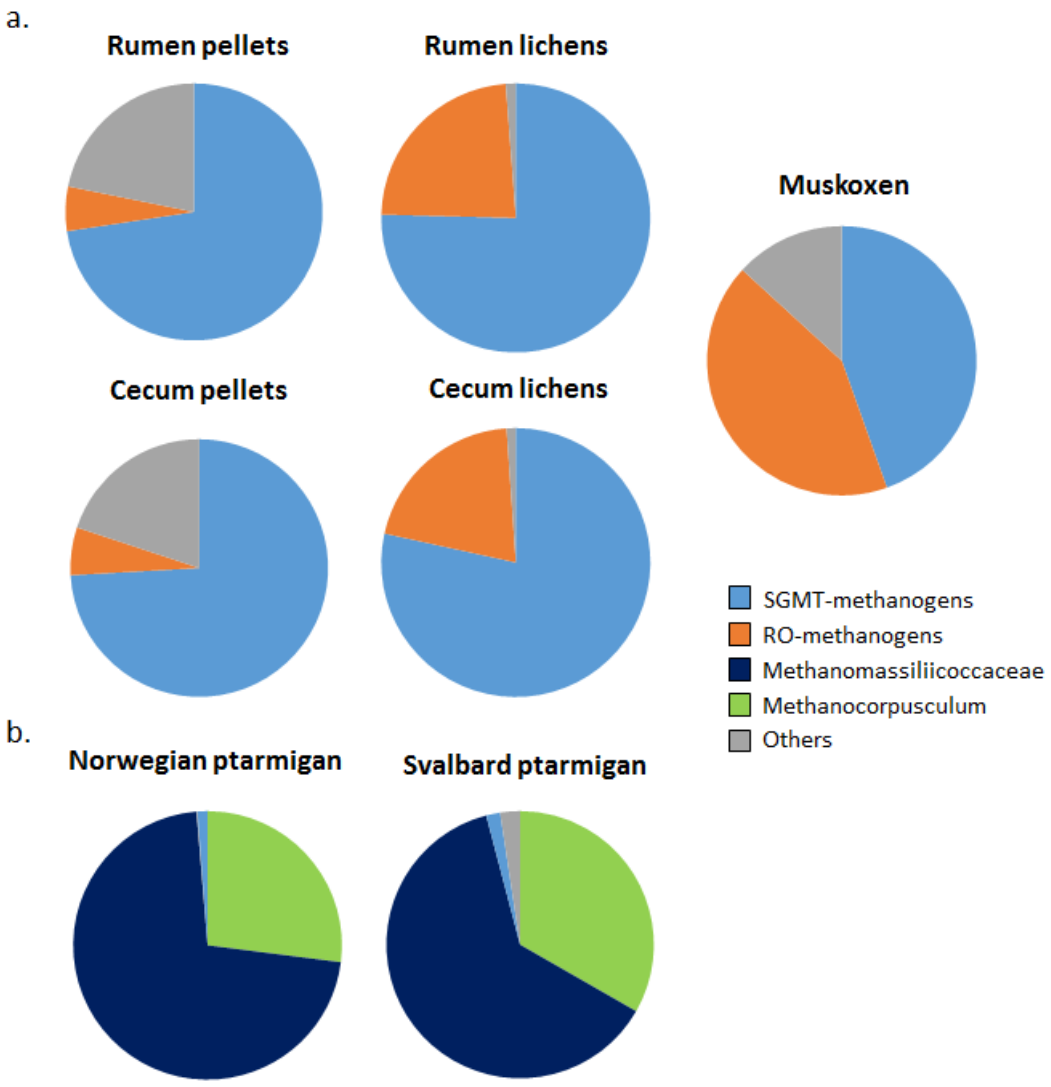

Fig. 7. Pie charts displaying the archaeal taxonomy from the Arctic herbivores investigated in this thesis. (A) Taxonomy of methanogens found in the rumen and cecum of Norwegian reindeer fed either lichens or pellets, in addition to muskoxen fed a graminoids-based diet. (B) Taxonomy of methanogens in wild ptarmigans from Svalbard and northern Norway. No information on the methanogens in captive ptarmigans was included due to the lack of amplicon sequencing studies with cecum samples from this group. Methanogens were classified according to King et al. [19] to allow a better comparison.

9.1.3. Effects of types of polysaccharides on archaeal profiles

The composition of carbohydrates present in the diet may also influence the gut archaeal profiles. *M. ruminantium* (RO-clade) has been positively related to diets high in fiber in wild moose [5], which concurs with the high presence of this clade of methanogens in muskoxen grazing on a graminoids-based diet (high in fiber) (**PAPER II**). In vascular plants such as grasses the cell wall is composed of cellulose, hemicellulose (including xylans and xyloglucans) and lignin. Noticeably, some species of lichens commonly eaten by Norwegian reindeer (e.g. *Cladonia* spp.) are high in hemicellulose [105]. It has to be noted that the hemicellulose fraction in lichens has not yet been fully characterized and presents some structural differences with hemicellulose from graminoids [137]. The "cell wall fraction" in the chemical analysis of lichens contains not only hemicellulose but also different lichen carbohydrates such as the lichen starch lichenin [137,138]. Despite such structural differences, it seems that the ingestion of a fibrous diet may be associated to an increase in RO-methanogens in both Norwegian reindeer fed lichens and muskoxen. Nonetheless, *M. ruminantium* was also found at a high proportion in lambs fed a corn-based diet (high in starch, low in fiber) [139], and cattle fed a corn-based diet [20], indicating these methanogens are not uniquely present with a specfic type of polysaccharides.

In ptarmigans, a more clear association between diet composition and archaeal profiles was observed. Dominance by *Methanomassiliicoccaceae* was related to the degradation of pectic compounds by other microbial groups, like bacteria from the genus *Bacteroides*, resulting in the production of methanol that may be further used by these methanogens (**PAPER III**). Association between diets high in pectin and prevalence of methanol-fueled methanogens was also observed in feces from the orangutan (*Pongo albelii*), but in this case with *Methanosphaera stadtamanae* as the major methanogens.

9.1.4. Effects of PSMs on bacterial profiles and methanogenesis

Methanogens (and methanogenesis) directly depend on byproducts resulting from the anaerobic fermentation by other microbial symbionts such as bacteria, protozoa and fungi. Accordingly, any changes in the community of these microbial groups related to the diet may potentially affect the taxonomy of methanogens, and therefore methanogenesis. The bacterial taxonomy for all the Arctic herbivores investigated in our studies are displayed in Fig. 8. As discussed with archaea, several factors such as PSMs contents or type of polysaccharides may affect the bacterial profiles. PSMs may present a heterogeneous effect on bacteria, but in general they have been associated to a negative reponse [56]. Some PSMs may directly bind to structural polysaccharides (e.g. cellulose, hemicellulose) thus hampering their degradation by

specialized bacteria. This aspect was discussed as the major reason for the decrease in the relative abundance of the fibrolytic *Ruminococcus* spp. in Norwegian reindeer fed lichens (Fig. 1; **PAPER I**), as some members of this genera have been described to efficiently degrade some lichen components such as hemicellulose and lichenin [140]. Noticeably, a direct relationship between digestibility of hemicellulose and CH_4 production has been reported in lactating cows fed forage [73]. Impoverished hemicellulose fermentation by effect of the ingestion of lichens (rich in PSMs) may reduce the production of substrates for methanogenesis thus leading to lower CH_4 yields in Norwegian reindeer fed lichens. In wild ptarmigans, although not related to methanogenesis, the ingestion of diets with presumably higher PSMs contents were discussed as an important factor leading to the differences between wild and captive ptarmigans (**PAPER III**). It was supported by the presence of genes involved in the degradation of phenolic compounds in addition to bacterial groups with reported PSMs-degrading properties (e.g. *Synergistetes, Actinobacteria,* etc) in wild but not captive ptarmigans. These findings suggest that dietary PSMs contents may exert a stronger effect on shaping the bacterial microbiota in comparison to methanogens.

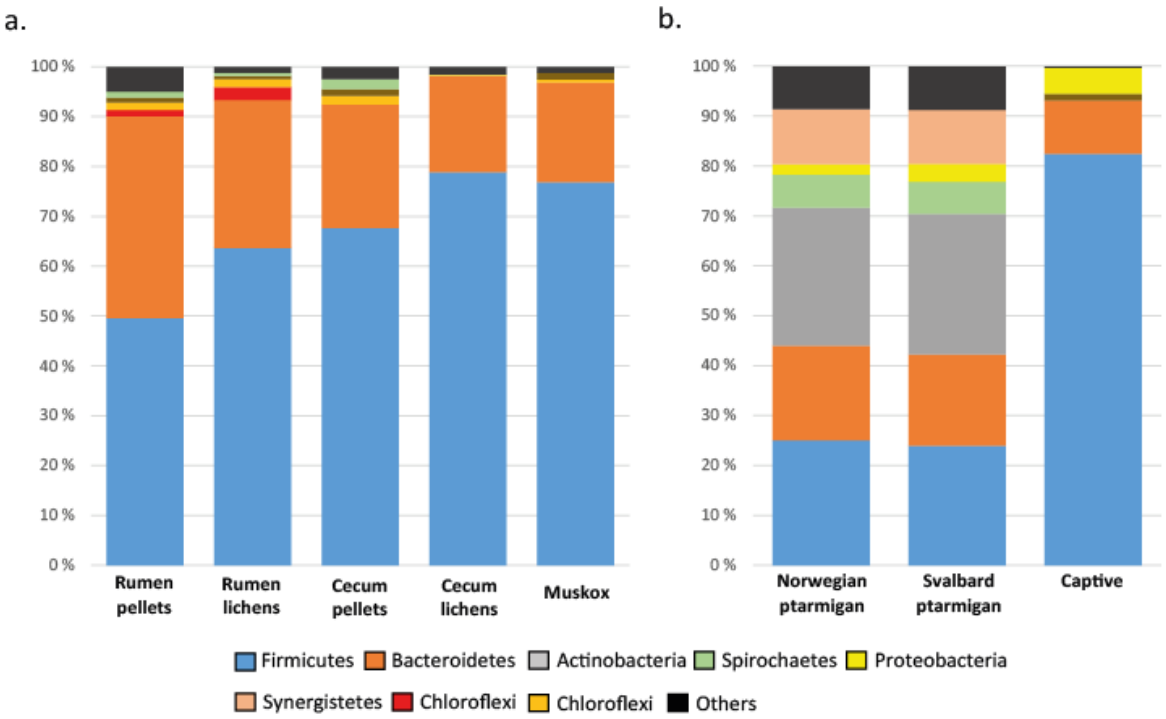

Fig. 8. Barr charts displaying the bacterial taxonomy from the Arctic herbivores investigated in this thesis. (A) Taxonomy of bacteria found in the rumen and cecum of Norwegian reindeer fed with lichens and pellets, in addition to muskoxen fed a graminoids-based diet. (B) Taxonomy of bacteria in wild rock ptarmigans from northern Noway (NptWd) and Svalbard (SptWd), and captive rock ptarmigans. Bacterial taxonomy was presented at phylum level.

Anaerobic bacterial PSMs degradation usually entails the production of H_2, which requires the co-operation between bacteria and other H_2-consuming microbes like methanogens [54]. Accordingly, the ingestion of diets high in PSMs would then lead to an increase of substrates like H_2 for methanogenesis derived from their degradation. This hypothesis would agree with the higher concentration of methanogens and genes related to methanogenesis in wild ptarmigans feeding on natural diets containing plants high in PSMs, but disagrees with the lower CH_4 yields reported to Norwegian reindeer fed lichens (compared to those fed pellet concentrates with presumably lower PSMs contents) [108]. The opposite process, i.e. anaerobic PSM-degradation requiring H_2 (instead of producing) has also been described for a particular bacterium (Eubacterium oxidoreducens) belonging to the class Clostridia [95]. The rumen microbiota in Norwegian reindeer has been reported to degrade some lichen PSMs like usnic acid [107], and PSM-tolerant bacteria belonging to the class Clostridia have been isolated from the rumen of Norwegian reindeer fed a mixed natural diet high in lichens [109,110]. The utilization of H_2 for PSMs degradation might then restrict its use by methanogens, which may potentially lead to reduced methanogenesis. Whether these PSMs-tolerant bacteria isolated from the rumen of Norwegian reindeer are also able to degrade such compounds, and their mechanism of action (i.e. either H_2-producing or H_2-consuming PSMs degradation), remains to be elucidated.

9.1.5. Effects of types of polysaccharides on bacterial profiles and methanogenesis

Similar to what was discussed to methanogenes, the type of structural polysaccharides may also influence the bacterial taxonomy, fermentation, and subsequently methanogenesis. *Firmicutes* dominated the rumen and cecum microbiota in Norwegian reindeer fed lichens and pellets as well as in feces of muskoxen fed a grass-based diet (high in fiber) (**PAPER I, PAPER II**). *Firmicutes* is a very diverse phylum encompassing bacteria displaying a broad range of physiological traits. Despite the heterogeneity of this phylum, they are commonly associated to the ingestion of diets high in fiber. Fiber fermentation has been associated to increased methanogenesis by increased H_2 and acetate production [59]. The ingestion of lichens (high in hemicellulose) and a graminoids-based diet (high in fiber) by Norwegian reindeer and muskoxen, respectively, may account for the dominance of *Firmicutes*, but it would not explain for the relatively low CH_4 output reported by these two ruminants [108,119]. In addition, *Firmicutes* (families *Lachnospiraceae* and *Ruminococcaceae*) was also dominant in captive ptarmigans fed a pelleted food (high in concentrates, low in fiber) (Table 3, **PAPER III**), and these captive birds were estimated to possess a lower CH_4 potential compared to wild ptarmigans based on their lower concentration of methanogens and relative abundances for

genes mediating methanogenesis. Overall, our results suggest a link between dominance by *Firmicutes* and potentially low methanogenesis in Norwegian reindeer, muskoxen and ptarmigans (captive), in contrast to previous studies reporting a positive correlation between *Firmicutes* and CH_4 emissions [141,142].

9.1.6. Role of the eukaryotic microbiota and methanogenesis

Due to the lack of reliable primers targeting the protozoal 18S rRNA gene at the time when the experiments were conducted, this project only covered the prokaryotic microbiota (methanogens and bacteria) in reindeer and muskoxen (**PAPER I-II**). In contrast, shotgun metagenomics with cecal samples from wild and captive ptarmigans allowed obtaining information on the eukaryotic fraction of this microbial consortium (**PAPER III**). Eukaryotic sequences were only identified in wild ptarmigans and they were mostly associated to *Parabasalia*, a class of flagellated protists from which several are recognized parasites to birds (e.g. genus *Trichomonas*), with little or no perceived influence in anaerobic fermentation (Table S3, **PAPER III**). Protozoa play an important role in the degradation of structural polysaccharides in the rumen of ruminants [30,31], and some groups (mostly holotrich) establish intimate associations with some type of methanogens thus providing them with H_2 and shelter [35,36]. About 37% of the enteric CH_4 is produced by protozoa-associated methanogens and the removal of these protozoa (defaunation) or the use of diets containing PSMs, which may negatively affect the population of protozoa, are common strategies to reduce methanogenesis [56,79]. Future efforts should be focused on describing the taxonomy of protozoa (and fungi) using specific primers targeting the eukaryotic 18S rRNA genes, which would allow overcoming the limitations of using shotgun metagenomics. It would help find out whether there are specific protozoa associated to methanogens and low methanogenesis as well as how their taxonomy is influenced by diet composition.

9.2. Functional role of the gut microbiota

9.2.1. Functional role of the bacterial microbiota in muskoxen and ptarmigans

As indicated, no previous information on the prokaryotic gut microbiome of muskoxen or wild ptarmigans (from Svalbard and northern Norway) existed. Our goal was to characterize the microbiota dwelling the gut to these arctic herbivores in order to better understand their digestive physiology and the ecological role of this microbiota in relation to their natural diets. Muskoxen possessed a bacterial fecal microbiota specialized in the degradation of a fiber-rich diet mostly associated to the phyla *Firmicutes* like family *Ruminococcaceae* (**PAPER II**). *Ruminococcaceae* encompasses several phylotypes involved in the degradation of structural

polysaccharides (e.g. cellulose), which may enable muskoxen to live off a highly fibrous diet like graminoids. However, fecal samples resembles the composition in the cecum, an anaerobic chamber where most of the undigested food material from the rumen is stored and degraded. It mainly consists of highly-fibrous material, a fact that may have biased the microbiota towards a high presence of fibrolytic bacteria. New analyses focused on investigating the rumen microbiota in muskoxen would help verify whether fibrolytic bacteria are also dominant, which may be assumed due to their natural diet high in fiber.

Wild rock ptarmigans from northern Norway and Svalbard presented a diverse bacterial microbiota composed of several phyla like *Actinobacteria, Firmicutes, Bacteroidetes, Synergistetes* and *Spirochaetes* (**PAPER III**). The presence of PSMs-degrading bacterial groups (e.g. *Synergistetes*, *Coriobacteriaceae*), and genes involved in the degradation of some PSMs (phenol / catechol) would enable ptarmigans to tolerate the ingestion of PSMs-rich plants as those they commonly feed on [143,144]. Although not reported in rock ptarmigans, some herbivores present endogenous mechanisms driving the direct detoxification of plant phytochemicals [145]. It would be necessary to investigate the existence of such mechanisms in ptarmigans as a complement to bacteria-driven PSM-degradation to fully comprehend the tolerance to plants high in PSMs observed in these birds.

The important representation of hydrolytic enzymes involved in the degradation of non-cellulosic polysaccharides presented by wild and captive ptarmigans would concurs with early studies indicating their capacity to select those parts of plants of high nutritional value (high in protein, low in fiber) [121,143,146]. Nonetheless, the major presence of enzymes mediating the depolymerization of hemicellulose indicates the consumption of plants with relatively high crude fiber contents (e.g. *Salix polaris* by Svalbard rock ptarmigans), which may account for the major available foodstuffs in certain periods like fall/winter [121,144]. These findings suggest a high versatility for the cecum microbiota of ptarmigans, which may be advantageous by enabling the consumption of foodstuffs with variable fiber contents during periods when high-quality food is scarce.

9.2.2. Functional role of the archaeal microbiota in Norwegian reindeer, muskox and ptarmigan

The ecological role played by the archaeal microbiota in these herbivores seems less intuitive than for bacteria. As introduced, CH_4 emissions from Norwegian reindeer fed lichens and muskoxen grazing graminoids were reported to be lower than for cattle (Table 3) [108,119]. Housing methanogens related to low CH_4 emissions, as suggested in this thesis for RO-

methanogens, might be beneficial for both ruminants to reduce their energy lossess. For instance, RO-methanogens increased in Norwegian reindeer fed lichens (Fig. 7), and lichens are mostly ingested by these ruminants during winter when strict energy conservation is required. Although the lichen-based diet used in our study does not completely resembles that naturally ingested by Norwegian reindeer as they also include vascular plants [102], it may give a first approximation on the composition of methanogens during that period and its potential ecological implications.

Muskoxen feces were collected during fall, a period when enough food supply is still available. Nonetheless, muskoxen mostly follow a fibrous diet throughout seasons with the exception of the short arctic summer. Although the results presented here may not be directly applicable to situations when food availability is scarce (e.g. winter), and therefore strict energy conservation is required, it suggests that methanogens with putative low CH_4 potentials are part of the gut microbiota in muskoxen. The potential ecological implications of housing these methanogens should, however, be considered with caution as we analyzed fecal samples and most of the enteric CH_4 in ruminants is produced in the large reticulo-rumen [134]. New analysis with samples collected from the rumen, and at different seasons are needed to verify the that these methanogens are commonly present in the gut of muskoxen as well as their potential ecological role.

Methanomassiliicoccaceae and *Methanocorpusculum* were dominant in wild ptarmigans (Fig. 7). It could not be possible to relate dominance by these methanogens with low CH_4 yields from ptarmigans, and, if so, it would indicate the opposite due to the higher CH_4 potential described for this group. Nonetheless, it has been estimated that around 5% of the daily energy requirement for rock ptarmigans comes from cecal fermentation [76], which may suggest that any potential loss of metabolic energy as CH_4 might be less decissive for ptarmigans than in large ruminants such as Norwegian reindeer and muskoxen.

10. Conclusions

This thesis provides and advancement of our understanding of the the physiology of the symbiotic gut microflora in three Arctic herbivores, Norwegian reindeer, muskox and rock ptarmigan, with special focus on methanogenesis and how this is related to diet composition (mainly high PSMs contens).

Overall, there was a trend between an increase in RO-methanogens and low CH_4 output in Norwegian reindeer and muskoxen, but it was not possible to link specific archaeal profiles

with low methanogenesis in ptarmigans. Whether increased of these methanogens was produced due to a direct effect by PSMs could not be established. This may indicate that other factors, e.g. the type and composition of structural polysaccharides, may exert a stronger effect on the archaeal profiles as observed in wild ptarmigans where dominance by *Methanomassiliicoccaecae* was mainly linked to the ingestion of pectin-rich foodstuffs via methanol production by pectin-degrading bacteria. This shows the complexity of attempting to relate specific archaeal profiles with diets high in PSMs accounting for low methanogenesis.

Although not fully understood, a potential link between higher presence of *Firmicutes* and low CH_4 yields was observed in the three different animals, in contrast to previous literature relating dominance by this bacterial group with higher methanogenesis.

Finally, muskoxen and wild ptarmigans possessed an specialized microbiota that may play an important role in the adaptation to their natural diets. Possessing methanogens that may result in low methanogenesis, such as in Norwegian reindeer, might be beneficial in order to keep low energy lossess in different periods.

11. Future perspectives

Despite the new findings presented in this study on the gut microbiome and the microbial digestion in three arctic herbivores (reindeer, muskox, and rock ptarmigans), more research is necessary to understand several unexplored areas that remain to be elucidated. We suggest that future research should focus on:

- Metagenomics and metatranscriptomics of rumen and cecum samples in reindeer fed lichens and pellets to search for differences in the active microbiota and expression levels of genes involved in methanogenesis to fully understand the microbial ecology of the methanogenesis in these arctic herbivores.
- Characterizing the eukaryotic microbiota (protozoa, fungi) using amplicon sequencing with primers targeting marker genes (18S rRNA, ITS) to search for potential groups associated to low methanogenesis. It would complete the knowledge on the gut microbiota in these arctic herbivores.
- Measuring the CH_4 emissions using alternative approaches (e.g. SF_6 technique for muskoxen; gas chromatography with ptarmigans) to obtain an accurate estimation of their CH_4 potentials.

- Characterizing the microbiome from muskoxen and ptarmigans at different seasons (spring/summer) to search for seasonal variations in their microbial profiles, and how is this microbiota shaped by variations in the diet composition.

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Paper I

Paper II

Paper III

Manuscript