CAMILA SOARES CUNHA

METHANE EMISSIONS IN DAIRY SYSTEMS: ANIMAL CATEGORY, PRODUCTION TRAITS AND RELATIONSHIP WITH MICROBIAL COMMUNITY

Thesis submitted to the Universidade Federal de Viçosa as partial fulfillment of the requirements of the Graduate Program in Animal Science to obtain the degree of *Doctor Scientiae.*

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APPROVED: July 18, 2016.

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DEDICATION

For my parents, Terezinha de Jesus Soares Cunha and Janio de Oliveira Cunha for all love, support, encouragement and teaching.

For my brother, Bruno Soares Cunha which is an example of joy.

For Fernando Henrique Carneiro Fortes Diniz, for all love, patience and for being a wonderful life partner.

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BIOGRAPHY

Camila Soares Cunha, daughter of Janio de Oliveira Cunha and Terezinha de Jesus Soares Cunha, was born in Indianópolis, São Paulo – Brazil on June 04, 1987.

She started the undergrad in Animal Science at Universidade Federal de Viçosa in 2006, and obtained a Bachelor of Science degree in Animal Science in 2010. In August of the same year, she started the Master's program in Animal Science at the same University, with major in physiology of animal production, submitting to the dissertation defense on July 18, 2012.

In November 2012, she started her Doctorate program in Animal Science, with major in physiology of animal production at the Universidade Federal de Viçosa. From January of 2015 to October of 2015 she was a visiting scholar at University of Wisconsin – Madison in the United States of America, where part of her research was developed.

On July 18th 2016, she submitted her dissertation to the thesis committee to obtain the Doctor Scientiae degree in Animal Science.

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ABSTRACT

CUNHA, Camila Soares, D.Sc. Universidade Federal de Viçosa, July, 2016. **Methane emissions in dairy systems: animal category, production traits and relationship with microbial community.** Adviser: Cristina Mattos Veloso. Co-adviser: Marcos Inácio Marcondes.

Rumen bacterial, archaeal and anaerobic fungal communities of Holstein dairy heifers and cows, in a tropical system of production, were characterized through sequencing the 16s rRNA and the ITS genes. In addition, we investigated the relationship between these communities and enteric methane (CH_4) emissions and productive traits, such as digestible dry matter intake (dDMI), digestible organic matter intake (dOMI), average body weight (BW), rumen pH, volatile fatty acids (VFA) and its main components, acetate, propionate and butyrate. Prepubertal heifers (PP), pubertal heifers (PB), and pregnant heifers (PG) were used in Chapter 1. Pregnant heifers emitted more CH_4 than others, followed by PB and PP. Regarding CH_4 emissions, the animals were split in high and low CH_4 emitters. Heifers were fed a diet composed by corn silage and concentrate (corn, soybean meal and minerals). *Prevotella*, *Ruminococcus*, *Coprococcus*, *Butyrivibrio*, *Clostridium*, *Shuttleworthia*, *SHD-231*, *CF231*, *p-75-a5, Methanobrevibacter*, *Methanosphaera* and *Caecomyces communis* were detected to be the core microbiome of the evaluated heifers. Families Bifidobacteriaceae and RF16 and genera *Acetobacter* and *Coprococcus* were strongly correlated with CH_4 emissions. Genera *Eubacterium*, *p-75-a5* and *SHD-231* showed inverse correlations with CH_4 emissions, dDMI, dOMI, BW and rumen pH. *Methanobrevibacter*, in archaeal community, and *Orpinomyces,* in anaerobic fungal, showed positive and weak correlations with CH_4 emissions. On the other hand, strong and negative correlations were observed among *Methanosphaera* and this variable. Prepubertal and PG heifers were the most divergent groups in relation to CH_4 emissions. Surprisingly, they did not differ in relative abundances of Firmicutes and Bacteroidetes, but PG had greater abundance of *Methanobrevibacter* and *Vadin CA11* and lower abundance of *Methanosphaera*. None of the bacterial, archaea and anaerobic fungi which correlate with CH_4 emissions showed significant correlations ($P>0.10$) with VFA and the individual concentrations of acetate, propionate and butyrate. Lastly, this work showed that bacterial, archaeal and anaerobic fungal communities did not covaried and the microbial communities did not covaried with

volatile fatty acids concentration either. In Chapter 2, high-producing (HP), medium-producing (MP), low-producing (LP) and dry (DC) were evaluated. The forage:concentrate ratios they were fed were 50:50 for HP, 70:30 for MP, 80:20 for LP, and 90:10 for DC. Considering the intake of digestible fraction of feed, DC emitted more CH_4, followed by MP, HP and LP, but the HP and LP emissions were similar. The core microbiome of the evaluated Holstein cows in tropical environment was composed by *Prevotella*, *Ruminococcus*, *Butyrivibrio*, *Clostridium*, *Coprococcus*, *Shuttleworthia*, *CF231*, *SHD-231*, *Methanobrevibacter,* and *Methanosphaera*. None of the anaerobic fungal operational taxonomic units (OTU) were found in all samples. Firmicutes and Bacteroidetes were the most abundant phyla found in the rumen of Holstein cows. For the archaeal community, *Methanobrevibacter* genera was the most abundant and in anaerobic fungi, most of the sequences were unclassified. The strongest negative correlations with CH_4 emissions detected were with the relative abundance of family Coriobacteriaceae and S24-7 and of genera *Butyrivibrio, Clostridium* and *Schwartzia*. Positive correlations were found between CH_4 emissions and families RF16 and Succinivibrionaceae. In the archaeal community, genera *Methanosphaera* relative abundance showed a strong negative correlation with CH_4. Surprisingly, no significant correlation between CH_4 emissions and *Methanobrevibacter* relative abundance was found. Relative abundance of genera *Vadin CA11* (in archaea) and *Caecomyces* (in anaerobic fungi) were detected to be positively correlated with CH_4 in g/day. Many families and genera from Firmicutes phylum showed positive correlations with dDMI and dOMI. None of the bacterial, archaea and anaerobic fungi which correlate with CH_4 emissions showed significant correlations ($P>0.1$) with VFA and the individual concentrations of acetate, propionate and butyrate. The most opposite results observed in the present study were among DC and HP. Dry cows showed greater CH_4 emissions in g/kg dDMI and g/kg of dOMI and, besides no differences were observed in relative abundances of Firmicutes, Bacteroidetes and Firmicutes:Bacteroidetes ratio, DC had lower relative abundance of Coriobacteriaceae, which was negatively correlated with CH_4, and greater relative abundance of Succinivibrionaceae, that was positively correlated with CH_4. In addition, DC had greater relative abundance of *Methanobrevibacter* and lower of *Methanosphaera*. Lastly, bacterial, archaeal and anaerobic fungal communities did no covary and VFA and microbial communities did not vary in a similar way either. Chapter 3 was

composed by two trials. In trial 1, CH_4 emissions were estimated from the seven previously described Holstein dairy cattle categories based on the SF_6 tracer gas technique and on IPCC (2006) equations. Enteric CH_4 emission was higher for the PP heifers when estimated by the equations proposed by the IPCC Tier 2. However, higher CH_4 emissions were estimated by the SF_6 technique for MP, HP and DC. Pubertal heifers, PG, and LP had equal CH_4 emissions as estimated by both methods. In trial 2, two dairy farms were monitored for one year to identify all activities that contributed in any way to GHG emissions. The total emission from Farm 1 was 3.21 t CO_2e/animal/yr, of which 1.63 t corresponded to enteric CH_4. Farm 2 emitted 3.18 t CO_2e/animal/yr, with 1.70 t of enteric CH_4. For the carbon balance calculations, when the carbon stock in pasture and other crops was considered, the carbon balance suggested that both farms are sustainable for GHG, by both estimation methods. On the other hand, carbon balance without carbon stock, by both estimation methods, suggests that farms emit more carbon than the system is capable of stock. It was concluded that IPCC estimations can underestimate CH_4 emissions from some categories while overestimate others. However, considering the whole property, these discrepancies were offset and we would submit that the equations suggested by the IPCC properly estimate the total CH_4 emission and carbon balance of the properties. Thus, the IPCC equations should be utilized with caution, and the herd composition should be analyzed at the property level.

RESUMO

CUNHA, Camila Soares, D.Sc. Universidade Federal de Viçosa, julho de 2016. **Emissões de metano em sistemas de produção de leite: categoria animal, características produtivas e relação com a comunidade microbiana.** Orientadora: Cristina Mattos Veloso. Coorientador: Marcos Inácio Marcondes.

As comunidades de bactérias, archaeas e fungos anaeróbios do rúmen de novilhas e vacas Holandesas, em um sistema de produção de leite em clima tropical foram caracterizadas. Além disso, a relação entre estas comunidades com a emissão de metano entérico (CH_4) e com características produtivas, como consumo de matéria seca digestível (CMSd), consumo de matéria orgânica digestível (CMOd), peso corporal médio (PC), pH ruminal, ácidos graxos voláteis (AGV) e seus principais constituintes, acetato, propionato e butirato. Novilhas pré-púberes (PP), púberes (PB) e em gestação (PG) foram utilizadas no trabalho do Capítulo 1. O grupo PG emitiu mais CH_4 que os demais, seguido por PB e PP. Em relação à emissão de CH_4, os animais foram divididos em alto em baixo emissores. As novilhas foram alimentadas com uma dieta composta por silagem de milho e concentrado (milho, farelo de soja e minerais). *Prevotella*, *Ruminococcus*, *Coprococcus*, *Butyrivibrio*, *Clostridium*, *Shuttleworthia*, *SHD-231*, *CF231* e *p-75-a5*, *Methanobrevibacter*, *Methanosphaera* e *Caecomyces communis* foram detectadas como o microbioma core das novilhas avaliadas. As famílias Bifidobacteriaceae e RF16 e gêneros *Acetobacter* e *Coprococcus* foram fortemente correlacionadas com as emissões de CH_4. Os gêneros *Eubacterium*, *p-75-a5* e *SHD-231* mostraram correlações inversas com emissão de CH_4, CMSd, CMOd, PC e pH ruminal. *Methanobrevibacter*, na comunidade de archaeas e *Orpinomyces*, dentre os fungos anaeróbios, mostraram correlações positivas e fracas com as emissões de CH_4. Por outro lado, correlações fortes e negativas foram observadas entre *Methanosphaera* e esta variável. Novilhas PP e PG foram os grupos mais divergentes em relação às emissões de CH_4. Inesperadamente, a abundância relativa de Firmicutes e Bacteroidetes não diferiram entre estes grupos, mas PG apresentou maior abundância relativa de *Methanobrevibacter* e *Vadin CA11* e menor abundância de *Methanosphaera*. Nenhuma das bactérias, archaeas e fungos anaeróbios que foram correlacionados com as emissões de CH_4 mostraram correlações significativas com AGV e com as concentrações individuais de acetato, propionato e butirato ($P>0.10$). Por fim, este trabalho mostrou que as comunidades ruminais de bactérias, archaeas

e fungos anaeróbios não covariaram entre si e que estas comunidades também não covariaram com a concentração de AGV. No Capítulo 2, vacas de alta (HP), média (MP) e baixa (LP) produção de leite e vacas secas (DC) foram avaliadas. As relações volumoso:concentrado utilizadas foram 50:50 para HP, 70:30 para MP, 80:20 para LP e 90:10 para DC. Considerando o consume da fração digestível do alimento, DC emitiu mais CH_4, seguida por MP, HP e LP, sendo que as emissões de HP e LP foram similares. O microbioma core das vacas Holandesas avaliadas em ambiente tropical, foi composto por *Prevotella*, *Ruminococcus*, *Butyrivibrio*, *Clostridium*, *Coprococcus*, *Shuttleworthia*, *CF231*, *SHD-231*, *Methanobrevibacter* e *Methanosphaera*. Nenhuma unidade taxonômica operacional (OTU) da comunidade de fungos anaeróbios foi encontrada em 100% das amostras. Firmicutes e Bacteroidetes foram os filos bacterianos mais abundantes encontrados no rúmen de vacas Holandesas. Na comunidade de archaeas, o gênero *Methonobrevibacter* foi o mais abundante e na comunidade de fungos anaeróbios a maioria das sequências foram de classificação indefinida. A correlação negativa mais forte com emissão de CH_4 foi com a abundância relativa das famílias Coriobacteriaceae e S24-7 e dos gêneros *Butyrivibrio, Clostridium* e *Schwartzia*. Correlações positivas foram encontradas entre as emissões de CH_4 e as famílias RF16 e Succinivibrionaceae. Na comunidade de archaeas, a abundância relative do gênero *Methanosphaera* apresentou uma forte correlação negativa com CH_4. Surpreendentemente, não foram observadas correlações significativas entre emissões de CH_4 e *Methanobrevibacter*. As abundâncias relativas dos gêneros *Vadin CA11* (dentre as archaeas) e *Caecomyces* (dentre os fungos anaeróbios) foram correlacionadas positivamente com CH_4 in g/day. Várias famílias e gêneros do filo Firmicutes apresentaram correlações positivas com CMSd e CMOd. Nenhuma das bactérias, archaeas e fungos anaeróbios que foram correlacionados com as emissões de CH_4 mostraram correlações significativas com AGV e com as concentrações individuais de acetato, propionato e butirato ($P>0.10$). Os resultados mais opostos observados neste trabalho foram entre HP e DC. Vacas secas apresentaram maior emissão de CH_4 em g/kg de CMSd e g/kg de CMOd e, apesar de não terem sido observadas diferenças nas abundâncias relativas de Firmicutes, Bacteroidetes e na relação Firmicutes:Bacteroidetes, DC apresentou menor abundância de Coriobacteriaceae, que foi negativamente correlationada com CH_4 e maior abundância de Succinivibrionaceae, que foi positivamente correlacionada com CH_4. Além disso, DC teve maior abundância relativa de

Methanobrevibacter e menor de *Methanosphaera*. Por fim, este trabalho mostrou que as comunidades ruminais de bactérias, archaeas e fungos anaeróbios não covariaram entre si e que estas comunidades também não covariaram com a concentração de AGV. O Capítulo 3 foi composto de dois ensaios. No ensaio 1, emissões de CH_4 das sete categorias de animais Holandeses previamente descritas utilizando a técnica do gás traçador hexafluoreto de enxofre (SF_6) e as equações propostas pelo Tier 2 do IPCC (2006). A emissão de CH_4 foi maior para PP quando estimada pelas equações do IPCC (2006). Entretanto, maiores emissões de CH_4 foram observadas para MP, HP e DC, quando estimadas pela técnica do SF_6. Os grupos PB, PG e LP tiveram emissões equivalentes quando estimadas pelos dois métodos. No ensaio 2, duas fazendas de gado de leite foram monitoradas por um ano para identificar todas as atividades que contribuíram, de alguma forma, para a emissão de gases de efeito estufa (GHG). A emissão total da Fazenda 1 foi 3,21 t CO_2e/animal/ano, dos quais 1,63 t corresponderam à emissão de CH_4 entérico. A Fazenda 2 emitiu 3,18 t CO_2e/animal/ano, dos quais 1,70 t foram CH_4 entérico. Para os cálculos de balanço de carbono, quando o estoque de carbono no pasto e em outras culturas foi considerado, o balanço de carbono sugeriu que ambas fazendas foram sustentáveis para a emissão de GHG, por ambos métodos de estimação. Por outro lado, o balanço de carbono sem o carbono estocado mostrou que as fazendas emitiram mais carbono que o sistema era capaz de estocar, por ambos métodos. Conclui-se que as equações do IPCC (2006) podem subestimar a emissão de CH_4 de algumas categorias e superestimar de outras. Entretanto, considerando a propriedade como um todo, as discrepâncias foram anuladas e pode-se dizer que as equações sugeridas pelo IPCC (2006) podem estimar apropriadamente a emissão total de CH_4 e o balanço de carbono de fazendas. Assim, as equações do IPCC (2006) devem ser utilizadas com cuidado, e a composição do rebanho deve ser levada em consideração.

GENERAL INTRODUCTION

Degradation of feed components in rumen is a coordinated process involving a great diversity of bacteria, archaea, anaerobic fungi and protozoa in order to converts plant materials into digestible compounds (Jami and Mizrahi, 2012). Thus, primary fermenters with hydrolytic activity, such as bacteria, protozoa and anaerobic fungi, break down proteins, starch and plant cell wall carbohydrates generating free amino acids and sugars (Boadi et al., 2004). Microbial protein, volatile fatty acids (VFA), hydrogen (H_2) and carbon dioxide (CO_2) are, then, produced from these simple products by primary and secondary digestive microorganisms (Boadi et al., 2004; Kittelman et al., 2014).

Because of the different pathways used in VFA production, different amounts of H_2 can be formed during degradation. Preferred pathways to produce acetic acid are the major source of H_2 (Van Soest, 1982; Boadi et al., 2004). However, H_2 generally do not accumulate in rumen because methanogenic archaea produce energy by reducing CO_2 with electrons from H_2 oxidation, generating methane (CH_4) (Kittelman et al., 2013). Thus, acetate and butyrate production leads to more CH_4 production (because it generates more available H_2) while propionate production pathway competes with methanogenesis for utilizing H_2 (Boadi et al., 2004). Since CH_4 has not nutritional value for the animal, most of it is released for the environment by eructation. Once released, CH_4 is an important greenhouse gas (GHG), with a global warming potential of 28 times CO_2 (King et al., 2011; IPCC, 2014). Besides, CH_4 represents an energy loss of 2 to 12% of the total energy ingested by the animals (Johnson & Johnson, 1995). Thus researchers have studied the whole ruminal microbiota and their interactions to achieve an integrated understanding of the methanogenic process and to draw

effective mitigation strategies to produce animal protein with a smaller environmental impact.

Several factors such as diet, stress, climate and geographical conditions affect the ruminal microbial community (Wright et al., 2004; Tajima et al., 2007; Zhou et al., 2009). The type of feed (Menezes et al., 2011) and quality of forage (Reis et al., 2016) that animals consume are key factors in modulating the availability of H_2 to be used by methanogens. A more fibrous diet leads to more residual H_2 because of the greater amounts of acetate produced. The same can be stated for a low quality forage. While diets based on high starch feeds induce a greater production of propionate, lowering the production of H_2, thus lowering CH_4 production.

Countries which are located in tropical climates often face challenges in dairy cattle production due to lower digestibility of forages and heat stress. Hence, co- and by-products of the digestive processes and the interactions between animals, environment, and feeds are substantially different than observed in animals raised in temperate climates (Kurihara et al., 1999). These peculiarities suggest a need for studies that can show the divergent points between different climates and geographical conditions.

Many studies have focused in sequencing the 16S rRNA gene of bacterial and archaeal communities (Cunha et al., 2011; Danielson et al., 2012; Jami and Mizrahi, 2012) but fewer researches looked for the anaerobic fungal communities (Kittelman et al., 2014). Anaerobic fungal community is expected to correlates with methanogenic, since fungi exerts crucial hole in degrading fibrous material (Kumar et al., 2015). Therefore, we believe that further studies are required to understand the relationships between methanogenesis and

ruminal bacterial, archaeal and anaerobic fungal communities, which could help in point out main microorganisms involved in CH_4 production process in rumen.

Intraruminal studies are extremely important to a better comprehension of the processes and to give the appropriate directions towards CH_4 emission mitigation actions. But, because of the increased concerns about global warming, to produce milk and meat at an environmentally sustainable livestock has become an obligation. So, it is necessary to look at GHG emissions at farm level and to develop inventories that can be used as an initial step to reduce these emissions and to advance in more sustainable practices.

Developing GHG inventories is important for determining the emission profile of the livestock sector; for the establishment of reduction targets; and to stimulate the adoption of sustainable alternatives in tropical climates (Santos et al., 2010). Despite their importance, the use of inventories on farms remains incipient and insufficient to provide more information of GHG emissions and carbon footprint estimation, which can be used as an environmental management tool when associated with parameters such as productivity, feed characteristics, feed intake and manure management system.

Enteric methane emissions need to be accounted for the farms inventories. However, estimating CH_4 emissions is not easy. Some estimation methods are the SF_6 tracer gas technique (Johnson et al., 1994) and the equations proposed by IPCC (2006). The SF_6 tracer gas technique is very laborious and return highly variable results (Vlaming et al., 2008). IPCC (2006) uses the equations proposed by the NRC (2001) to calculate gross energy intake, which were developed based on temperate climate data and, therefore, we hypothesize that it could cause distortions when utilized in tropical environment. Thus, comparing these estimation methods for different categories in tropical dairy systems and the effects that using

these estimates can cause in carbon balance calculation is crucial to improve the quality of inventories of GHG emissions and carbon balance estimations.

References

Boadi, D., Benchaar, C., Chiquette, J. and Massé, D. (2004). Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Can. J. Anim. Sci. 84: 319–335.

Cunha, I.S., Barreto, C.C., Costa, O.Y.A., Bomfim, M.A., Castro, A.P, Kruger, R.H., Quirino, B.F. (2011). Bacteria and Archaea community structure in the rumen microbiome of goats (Capra hircus) from the semiarid region of Brazil. Anaerobe, 17:118-124.

Danielson, R., Schnürer, A., Arthurson, V., Bertilsson, J. (2012). Methanogenic population and methane production in Swedish dairy cows fed different levels of forage. Appl. Environm. Microbiol., 78 (17): 6172-6179.

IPCC (2006) Intergovernmental panel on climate change: Emissions from livestock and manure management. In: Eggleston S., Buendia L., Miwa K., Ngara T. and Tanabe K. (eds.) IPCC Guidelines for National Greenhouse Gas Inventories, pp. 10.1-10.84. Hayama, Japan: Institute for Global Environmental Strategies.

Intergovernmental Panel on Climate Change. Climate change 2014. Synthesis report 2014.

Jami, E., Mizrahi, I. (2012). Composition and similarity of bovine rumen microbiota across individual animals. PLoS ONE 7(3): e33306.

Johnson, K.A., Huyler, M., Westberg, H.H., Lamb, B. and Zimmerman, P., 1994. Measurement of methane emissions from ruminant livestock using a sulphur hexafluoride tracer technique. Environ. Sci. Technol. 28:359–362.

Johnson, K.A., Johnson, D.E. (1995). Methane emissions from cattle. J. Anim. Sci. 73:2483-2492.

King, E.E., Smith, R.P., St-Pierre, B., Wright, A.D.G. (2011). Differences in the rumen methanogen populations of lactating Jersey and Holstein dairy cows under the same diet regimen. Appl Environ Microbiol.77: 5682–5687.

Kittelmann, S., Seedorf, H., Walters, W.A., Clemente, J.C., Knight, R., Gordon, J.I., Janssen, P. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of

bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. PLoS ONE 8(2): p. e47879.

Kittelmann, S., Pinares-Patiño, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., McEwan, J.C., Janssen, P. (2014) Two Different Bacterial Community Types Are Linked with the Low-Methane Emission Trait in Sheep. PLoS ONE 9(7): e103171.

Kumar, S., Indugu N., Vecchiarelli, B., Pitta, D.W. (2015). Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to changes in diet and age in the rumen of dairy cows. Front. Microbiol. 6:781.

Kurihara, M., Magner, T., Hunter, R. A., McCrabb, G. J., 1999. Methane production and energy partition of cattle in the tropics. Br. J. Nutr. 81, n.3, p.227-234.

Menezes, A.B., Lewis, E., O'Donovan, M., O'Neill, B.F., Clipson, N., Doyle, E.M. (2011). Microbiome analysis of dairy cows fed pasture or total mixed ration diets. FEMS Microbiol. Ecol.78, 256–265.

National Research Council - NRC. Nutrient requirements of dairy cattle. 7.rev.ed. Washinton, D.C.: 2001. 381p.

Reis, W.L.S., Detmann, E., Batista, E.D., Rufino, L.M.A., Gomes, D.I., Bento, C.B.P., Mantovani, H.C., Valadares Filho, S.C. (2016). Effects of ruminal and post-ruminal protein supplementation in cattle fed tropical forages on insoluble fiber degradation, activity of fibrolytic enzymes, and the ruminal microbial community profile. Animal Feed Science and Technology. 218, 1-16.

Santos, D.R.R., Picanço, A.P., Maciel, G.F. and Serra, J.C.V., 2010. Study of greenhouse gases offsets of the Federal University of Tocantins State, Brazil – academic senate and campus of the University of Palmas: A way of environmental mitigation. Revista Geográfica Acadêmica, 4, 29-40.

Tajima, K., Nonaka, I., Higuchi, K., Takusari, N., Kurihara, M., Takenaka, A., Mitsumori, M., Kajikawa, H., Aminov, R.I. (2007). Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. Anaerobe 13:57-64.

Van Soest, P. J. (1982). Gastrointestinal fermentations: in Nutritional ecology of the ruminant. Ruminant metabolism, nutritional strategies, the cellulolytic fermentation and the chemistry of forages and plant fibers. Cornell University Press, Ithaca, NY. Pages 152–177.

Vlaming, J.B., Lopez-Villalobos N., Brookes, I.M, Hoskin, S.O., Clark, H. (2008). Within and between animal variance in methane emissions in non-lactating dairy cows. Australian Journal of Experimental Agriculture, 48, 124-127.

Wright, A.D.G., Williams, A.J., Winder, B., Christophersen, C.T., Rodgers, S.L., Smith, K.D. (2004). Molecular diversity of rumen methanogens from sheep in Western Australia. Applied and Environmental Microbiology. 70:3, 1263-1270.

Zhou, M., Hernandez-Sanabria, E., Guan, L.L. (2009). Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Applied and Environmental Microbiology. 75:20, 6527-6533.

CHAPTER 1

METHANE EMISSIONS AND PRODUCTION TRAITS OF HOLSTEIN HEIFERS RAISED IN TROPICAL CONDITIONS AND THEIR RELATIONSHIPS WITH BACTERIAL, ARCHAEAL AND ANAEROBIC FUNGAL RUMEN COMMUNITIES

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Abstract

Rumen bacterial, archaeal and anaerobic fungal communities of Holstein dairy heifers in a tropical system of production were characterized through sequencing the 16s rRNA and the ITS genes. In addition, we investigated the relationship between these communities and enteric methane (CH_4) emissions and productive traits such as digestible dry matter intake (dDMI), digestible organic matter intake (dOMI), average body weight (BW), rumen pH, volatile fatty acids (VFA) and its main constituents, acetate, propionate and butyrate. Prepubertal heifers (PP), pubertal heifers (PB), and pregnant heifers (PG) were used in this study. Regarding CH_4 emissions, the animals were split in high and low CH_4 emitters. Pregnant heifers emitted more CH_4 than others, followed by PB and PP. Heifers were fed a diet composed by corn silage and concentrate (corn, soybean meal and minerals). Core microbiome of the evaluated heifers was composed by *Prevotella*, *Ruminococcus*, *Coprococcus*, *Butyrivibrio*, *Clostridium*, *Shuttleworthia*, *SHD-231*, *CF231*, *p-75-a5*, *Methanobrevibacter, Methanosphaera*, and *Caecomyces communis*. Pubertal heifers bacterial community was found to be more diverse than PP and PB anaerobic fungal community richer than PP, probably due to changes in microbiota towards a mature composition. Differences in richness and diversity of the communities were not observed in relation to CH_4 emission levels groups. Families Bifidobacteriaceae and RF16 and genera *Acetobacter* and *Coprococcus* were strongly correlated with CH_4 emissions. Genera *Eubacterium*, *p-75-a5* and *SHD-231*, which are not usually discussed in terms of their role in ruminal fermentation, showed inverse correlations with CH_4 emissions, dDMI, dOMI, and BW. *Methanobrevibacter*, in archaeal community, and *Orpinomyces,* in anaerobic fungal, showed positive weak correlation with CH_4 emissions. On the other hand, strong and negative

correlations were observed among *Methanosphaera* and this variable. Prepubertal and PG heifers were the most divergent groups in relation to CH_4 emissions. Surprisingly, they did not differ in relative abundances of Firmicutes and Bacteroidetes, but PG had greater abundance of *Methanobrevibacter* and *Vadin CA11* and lower abundance of *Methanosphaera*. The same trend was followed by the comparison among low and high CH_4 emitters. None of the bacterial, archaea and anaerobic fungi which correlate with CH_4 emissions showed significant correlations ($P>0.1$) with VFA and the individual concentrations of acetate, propionate and butyrate. Lastly, this work showed that bacterial, archaeal and anaerobic fungal communities did not covaried and the microbial communities did not covaried with volatile fatty acids concentration either.

1. Introduction

Rumen undergoes anatomical and physiological changes from the newborn rumen to a mature cow rumen (Beharka et al., 1998). These modifications are driven by factors such as diet, age and ruminal microbial composition (Anderson et al., 1987; Beharka et al., 1998; Jami et al., 2013). The main diet and ruminal microbial composition effect is due to the different kinds and proportion of volatile fatty acids produced in degradation of feed components, and their role in the wall villi development during animal growth (Beharka et al., 1998). Evidences that rumen microbiota vary with physiological stages are supported by Jami et al. (2013). Their research concluded that rumen microbiota of animals grouped in 1-3 days old, two months old, six months old and two years old were substantially different, even among groups that received the same diet.

During degradation in rumen, most of the methanogens (archaea) use hydrogen (H_2) and carbon dioxide (CO_2) released by other microorganisms to produce methane (CH_4)

(Stewart et al., 1997). Besides being one of the greenhouse gases and highly polluting - 28 times more global warming potential than carbon dioxide (IPCC, 2014) - CH_4 represents an energy loss of 2 to 12% of the total energy ingested by the animals (Johnson & Johnson, 1995). Thus researchers have studied the ruminal microbiota and their interactions to achieve an integrated understanding of the methanogenic process and to develop effective mitigation strategies to produce animal protein with a smaller environmental impact.

It has been demonstrated before that heifers of distinct live weights emitted different methane amounts (Jiao et al., 2014) and, since methanogenesis is dependent on the fermentation activity of the rumen microorganisms, we hypothesize that differences in rumen microbiota of heifers from different physiological stages (age and live weight) may help in explaining different CH_4 emissions among categories feed the same diet.

Countries which are located in tropical climates often face challenges in dairy cattle production due to lower digestibility of forages and heat stress. Hence, co- and by-products of the digestive processes and the interactions among animals, environment, and feeds are substantially different than observed in animals raised in temperate climates (Kurihara et al., 1999). These peculiarities suggest a need for studies that can show the divergent points between different climates and geographical conditions. To our knowledge, scientific works that have characterized ruminal microbiota from Holstein heifers in tropical environment and have associated the microbiota with CH_4 emissions are scarce.

This study aimed to investigate the relationship between ruminal microbial community and enteric CH_4 emissions from dairy heifers from different physiological stages, in a tropical production system.

2. Material and methods

2.1. Animals and management

This study was carried out at the Unit for Teaching, Research, and Extension on Dairy Cattle of the Universidade Federal de Viçosa (UFV), Brazil and at the Microbial Sciences Department at University of Wisconsin, USA. The project was approved by the Ethics Committee in the Use of Production Animals from the UFV (protocol number 99/2014).

Heifers from different physiological stages were split into three groups according to their average body weight and gestational condition, as follows: prepubertal heifers (PP), 204±14.7 kg (n=5), pubertal heifers (PB), 358±11.9 kg (n=4) and pregnant heifers (PG), 473±23.6 kg (90 days post artificial insemination, n=5).

Heifers were kept under the same feeding management to which they were already adapted in the production system and were housed in individual tie stalls. Heifers' diet was constituted by 90% of corn silage and 10% of concentrate based on corn, soybean meal, and minerals (Table 1). Animals were fed twice a day, at 7 am and 3 pm and had *ad libitum* access to water. Feed was adjusted daily to keep the orts between 5-10% of the total provided. At the beginning and end of the trials the animals were weighted without fasting, always before morning feeding to estimate the average body weight.

The trials were performed separately for each group. The first five days of each trial were for adaptation of the animals to tie stall housing and yokes used for CH_4 evaluation. Gas collections from animals' eructation for evaluation of CH_4 emissions began on the sixth day. These collections started at 7 am through 24 hours and were performed, at least, up to the 10^{th} day. Since the experiment was designed to obtain five high quality gases samples of each animal, in case of problems in the sampling process, the collection of that day was

repeated after day 10. Evaluation of voluntary intake was performed from day 6 to 10, feces samples were taken in day 6 and rumen samples, in day 10.

2.2. Food, orts and feces sampling

Through five days, starting at day 6 in each trial, forage and orts of each animal were sampled. The amounts of food provided and the weight of the orts were registered. Concentrate ingredients were sampled during mixing. At day 6, feces were collected during a 24 hour period. Food, orts and feces samples of each animal were grouped per trial, and were analyzed for dry matter (DM; Detmann et al., 2012; method INCT-CA nº G-003/1), crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), non-fiber carbohydrates (NFC) and ashes (MM) contents (AOAC, 1990) and the results were used for the calculation of the dry matter intake (DMI), dry matter digestibility (DMD), organic matter intake (OMI) and organic matter digestibility (OMD).

2.3. Methane emissions measurements

The tracer gas sulfur hexafluoride (SF_6) technique (Johnson et al., 1994) and the adaptations proposed by Primavesi et al. (2004) were used for the evaluation of CH_4 emissions by the animals. Briefly, the flow of CH_4 released by the animal was calculated in relation to the flow of SF_6, measured from the SF_6 release rate from the permeation capsule lodged in the rumen and from the concentrations of CH_4 and SF_6 in gas samples (Johnson et al., 2007). On the 4^{th} day of each trial, the animals were forced to ingest one permeation capsule with known SF_6 release rate. To collect the gases, a PVC-tube collection-container yoke was used with a halter coupled to a collecting apparatus. Before the beginning of each collection day, the yokes went through a cleaning process, were evacuated at approximately

–14 PSI, and then placed on the neck of each animal and coupled to the halter using a quick attachment. After 24 h, the yokes that were on the animals were switched for a cleaned and evacuated yokes, and the pressure of each yoke was recorded. The yokes containing samples of gases were pressurized with approximately 14 PSI of N_2 gas 99.999% purity. After a period of at least 1 h for homogenization of gases inside the yoke, the pressure was recorded once again, and samples were collected in previously evacuated Exetainer® tubes.

Analysis of CH_4 and SF_6 levels were performed at the Laboratory of Gas Chromatography of EMBRAPA Dairy Cattle, in Juiz de Fora, Minas Gerais, Brazil. The concentrations of CH_4 and SF_6 in the samples were evaluated in gas chromatograph. For SF_6 analysis, the detector μECD was used. The sampler system was bounding a 0.5 mL loop to a valve. In let typo split-splitless was used in splitless mode at 250 ºC. The separation system was composed by a column Molesieve 30 m × 0.530 mm × 25.0 μm, using gas N_2 as carrier gas at 5.0 mL/min in the column. Certified standards were used in the following concentrations for calibration of the chromatograph: 30, 100, 500; 1500 and 3000 ppt. For CH_4 analysis, a chromatograph equipped with two 6-way valves was used. One of the valves was used for the sampler system and the other one was used as a selector, allowing or not the passage of the constituents through the second column. In let typo split-splitless was used in splitless mode at 120 ºC. Separation system was composed by two columns (HP-Plot/Q 30m × 0.530 mm × 40.0 m and HP-Molesieve 30 m × 0.530 mm × 25.0 μm), using H_2 as carrier gas at a flow rate of 7.0 mL/min. Detector TCD and FID were used. The calibration was performed using certified standards in the concentrations 4.8, 9.7, 19.6, 102 and 203 ppm. The results were expressed in g of CH_4/animal/day, g of CH_4/kg of DMI, g of CH_4/kg of digestible DMI (dDMI) and g of CH_4/kg of digestible OMI (dOMI).

2.4. Ruminal sampling and fermentation pattern

At the 10th day of each trial, ruminal content was sampled four hours after the feeding time using the stomach tube technique (Lodge-Ivey et al., 2009; Henderson et al., 2013; Kumar et al., 2015) and the initial 200 mL of collected fluid were discarded, to avoid saliva contamination. Because of the collection method, rumen samples represented the liquid phase of ruminal content. A sample of approximately 70 mL was filtered through four layers of cheesecloth and split in two aliquots. The first one was destined for DNA extraction (50 mL), and the other one (20 mL), for volatile fatty acids (VFA) concentration analysis. Both samples were frozen at -80ºC until the time they were analyzed.

Volatile fatty acids (VFA) concentrations were analyzed using high-performance liquid chromatography (HPLC). Briefly, after a centrifugation step, 1.5 mL of a cell-free supernatant was treated as described by Siegfried et al. (1984) and analyzed in a chromatograph Dionex Ultimate 3000 Dual with a refractive index detector Shodex RI-101 at 45 ºC, using an ion-exclusion column Phenomenex Rezex ROA, 300 x 7.8 mm, also at 45 ºC. The mobile phase was H_2SO_4 (5 mM) at a flow rate of 0.7 mL/min.

2.5. DNA extraction, amplicon library preparation and sequencing

DNA extractions were performed as described by Stevenson & Weimer (2007) and then treated with RNAse Ribonuclease A (10 mg/mL). Total DNA was quantified using a Qubit® 2.0 Fluoremeter (Invitrogen, San Diego, CA, USA). Two Polymerase Chain Reactions (PCR) were performed. The primers used aimed V3 and V4 regions of the 16S ribosomal RNA gene (16S rRNA) for bacteria, the regions V6 and V8 of the same gene for achaea, and the ITS1 gene for anaerobic fungi (Kittelmann et al., 2013).

The first PCR aimed to amplify region of interest using specific primers with Illumina specific overhand adapters attached and the second one to attach dual indices and Illumina sequencing adapters. The reactions were made under the following conditions: 95ºC for three minutes, 25 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds and 72ºC for five minutes for the first PCR reaction and 95ºC for three minutes, eight cycles of 95ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds and 72ºC for 5 minutes for the second PCR reaction. These PCR were performed using 5-200 ng DNA, 0.5 µL of each primer (forward and reverse), master mix Kapa Biosystems® (Wilmington, MA, United States) and ultrapure water, totalizing a 25 µL reaction. Between both PCR reactions a clean-up step was performed using an Invitrogen Pure Link Pro96 PCR Purification Kit. The products of the second PCR were loaded in a AquaPor LM low-melt agarose and the gel extraction was performed using the ZR-96 Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). DNA content of each sample was then quantified using a Qubit® 2.0 Fluoremeter, diluted, and pooled to 2 nM concentration solution. The library was denaturated at 96ºC using NaOH 0.2 N, then diluted with Hybridization Buffer to 20 pM, and combined with the PhiX control library (20 pM). Then, the library was loaded in the Illumina MiSeq cartridge and the sequencing run at Illumina MiSeq 2x300bp was performed as indicated at the manufacturer's guidelines.

2.6. DNA sequence processing analysis

The programme MOTHUR, version 1.36.1 (Schloss et al., 2009), was used to analyze the obtained libraries. Sequences that were not aligned, chimera sequences and all contaminants were removed. The sequences from bacteria and archaea were aligned against the SILVA 16S rRNA gene database and classified using the Greengenes database. For

anaerobic fungal analysis, sequences were classified using the Unit database and contaminants were removed as well. Sequence similarities greater than 97% were used as cut-offs to classify operational taxonomic units (OTU) at genus level. All sequences used for this study were publicly deposited in the NCBI Short Read Archive.

2.7. Microbial communities' analysis

Relative abundances were calculated as the number of sequences of the taxa divided by the number of total sequences present in the sample, times 100. Alpha diversity indexes for richness and diversity, Chao 1 and Shannon, and Good's Coverage were calculated in MOTHUR version 1.35.0 (Schloss et al., 2009).

2.8. Statistical analysis

The statistical programme R (R Core Team, 2015) was used to test differences in CH_4 emissions from each group using ANOVA, followed by a Tukey test, when necessary. Based on CH_4 emissions data, heifers were also split in groups of CH_4 emissions level (high or low) using NbClust package (Charrad et al., 2014) in R. Vegan package (Oksanen et al., 2015) was used to test differences in richness and diversity of bacterial, archaeal and anaerobic fungal communities when data were grouped into physiological groups or grouped into CH_4 emission level (CH_4G). Non-parametric tests were used to test de differences in relative abundances. Kruskal-Wallis (Agricolae package; Mendiburu, 2016) test was used for physiological groups and the Wilcoxon Rank Sum Test was used for testing the differences between CH_4G. For these tests, $P<0.10$ were accepted as significantly different.

Bacterial, archaeal and anaerobic fungal communities were visualized using a non-metric multidimensional scaling (nMDS) plots of the Bray-Curtis dissimilarity index. Non-metric multidimensional scaling was performed using Vegan package.

Analysis of Similarity test (ANOSIM; Clarke, 1993) was performed to access the significant difference in microbial community and groups (PP, PB and PG heifers and CH_4G). Similarity percentage analysis (SIMPER; Clarke & Warwick, 1994) was used to evaluate the contribution of each OTU on the differences seen in ANOSIM. To investigate if microbial community data and VFA concentrations changed in the same mode, a MANTEL test (Mantel, 1967) was performed. MANTEL was also used to evaluate if bacterial, archaeal and anaerobic fungal communities varies in a similar manner. All these analyses were performed with the aid of Vegan package in R. For these tests, p-values lower than 0.05 were accepted as statistically different.

Spearman correlation was used to correlate continuous variables, [CH_4 emissions, digestible dry matter intake (dDMI), digestible organic matter intake (dOMI), average body weight (BW), total volatile fatty acids (VFA) and concentrations of acetate, propionate and butyrate] and bacterial, archaeal and anaerobic fungal families and genera relative abundances using the corrplot R package (Wei, 2012). Family or genus level that showed significant correlations at $P<0.1$ for at least one variable were included in the correlogram.

3. Results

3.1. Methane emissions and production parameters

Methane emissions (Table 2) in g/day from PP heifers were lower ($P<0.01$) than CH_4 emissions in g/day from PB and PG heifers. Pubertal heifers showed greater CH_4 in g/day

(P>0.10) than PG heifers. When expressed in g/kg of DMI, PG heifers had higher CH_4 emission than PP (P<0.01) and PB (P<0.05). Pubertal heifers showed higher CH_4 in g/kg of DMI than PP heifers (P<0.05). Considering the digestible fractions of DMI and OMI, PG had greater CH_4 emission than PP (P<0.01). Methane emissions in g/kg of dDMI from PB were not different than PP and PG, and in g/kg of dOMI, PB and PG did not show differences in their emissions and PP emitted less than PB (P<0.10) and PG (P<0.01).

3.2. Sequencing and coverage metrics

After all filtering analyses using MOTHUR (v.1.35.0), the number of sequences reached was 164,421 for bacteria (mean= 11,744), 59,645 for archaea (mean= 4,260), and 9,039 for anaerobic fungi (mean= 646). For all amplicons, Good's Coverage of each sample was around 0.99.

3.3. Ruminal communities' composition

For the bacterial community, 21 phyla were found, but only 9 were present in all animals. The most abundant phyla were Firmicutes (51.27%), Bacteroidetes (26.44%), TM7 (11.67%), Proteobacteria (2.49%), and SR1 (2.36%) (Figure 1). The remaining phyla had relative abundance lower than 2% each. Within Firmicutes, 51.13% of the sequences did not reach the genus level, 25.34% were unclassified, 9.88% of the sequences belong to genus *Ruminococcus*, 4.50% to *Butyrivibrio*, 3.03% to *Coprococcus*, and 2.50% to *Clostridium*. The remain genera were responsible for percentages lesser than 1%. In the Bacteroidetes phylum, 60.78% of the sequences belonged to the genus *Prevotella*, 4.07% to genus *CF231*

(family Paraprevotellaceae), 24.34% of the sequences did not reach the genus level, and 8.01% were unclassified sequences.

All archaeal community sequences were attributed to the phylum Euryarchaeota. The relative abundance at genus level was greater for *Methanobrevibacter* (95.03%), followed by *Methanosphaera* (4.32%) (Figure 2). *Vadin CA11* and *Methanomicrococcus* had relative abundances lower than 1% (0.62% and 0.03%, respectively).

For the anaerobic fungal community, 90.23% of the sequences belonged to the phyla Chytridiomycota, and 9.77% were unclassified sequences. At genus level, the relative abundance was 52.23% for unclassified sequences, 45.36% for *Caecomyces*, 2.04% for *Orpinomyces* and 0.37% for *Neocallimastix* (Figure 3).

3.4. Communities richness and diversity

The number of bacterial species (Chao1 richness index) and the distribution of the taxa across all samples (Shannon diversity index) from each group were not different between animals that produced high or low CH_4. Pubertal heifers had a greater Shannon index for bacterial community than PP heifers and a greater Chao1 index for anaerobic fungal community was found for PB in relation to PP heifers. These indexes were not different between the archaeal community of PP, PB and PG heifers.

3.5. Core microbiome

The analysis of each bacterial OTU across all samples showed that 26% of the OTU are shared by only 1 to 10% of samples, 15% are shared for 10 to 20% and 22% of the OTU are present at the same time at 20 to 30% of samples. Thus, 63% of the OTU are being shared

for a small part of the sampled animals. Four percent or 38 of the OTU were find in 100% of the samples. At genus level, it can be highlighted the genera *Prevotella*, *Ruminococcus*, *Coprococcus*, *Butyrivibrio*, *Clostridium*, *Shuttleworthia*, *SHD-231*, *CF231* and *p-75-a5*.

Archaeal genera shared among all samples were *Methanobrevibacter* and *Methanosphaera*. For anaerobic fungal community, only one OTU appeared in 100% of the samples. This OTU was affiliated as the species *Caecomyces communis*.

3.6. Influence of physiological and production traits in communities' composition and structure

Bacterial and anaerobic fungal communities' composition did not differ ($P<0.05$) among PP, PB and PG groups and between high and low CH_4 emitters. Archaeal community was distinct by physiological stage groups ($P<0.05$) and by CH_4G ($P<0.01$). Basically, three OTU were detected in SIMPER as responsible for the changes in archaeal community, either for growing groups and CH_4 emission level groups (Table 3). These OTU were assigned as the genera *Methanobrevibacter* and *Methanosphaera*. Genus *Vadin CA11* was appointed as the responsible for 25% of the changes in archaeal community from PB and PG heifers. Abundance of genus *Methanosphaera* was different ($P<0.05$) between PP, PB and PG and it is clear that its abundance decreases as heifers become older and have higher body weight (Figure 2). Abundance of *Methanosphaera* was 1.85 times greater in low CH_4 emitter heifers ($P<0.01$) and abundance of *Vadin CA11* was 6.94 times greater in high CH_4 emitters ($P<0.1$). *Methanobrevibacter* abundance was only 1.02 times greater in high CH_4 emitters, nonetheless this difference was significant ($P<0.1$).

A non-metric multidimensional scaling (nMDS) was plotted using Bray-Curtis dissimilarity index matrices to show how microbial community structure influences CH_4

emissions, dDMI, dOMI, BW, and acetate:propionate (Figure 4). Environmental variables were significant in clustering animals in bacterial community only, thus figures of archaeal and anaerobic fungal communities were omitted.

3.7. Correlation among bacterial, archaeal and anaerobic fungal families/genera and physiological and production traits

A correlation matrix was created to correlate bacterial, archaeal and anaerobic fungal families and genera and the variables evaluated in this study (Figure 5). Several bacterial OTU showed to be correlated with variables such as CH_4 emissions, dDMI, dOMI, and BW. Eight families/genera were correlated with CH_4 emissions in all evaluated units (g/day, g/kg of DMI, g/kg of dDMI and g/kg of dOMI), among them, family Bifidobacteriaceae and the genera *Acetobacter*, *Coprococcus*, *Eubacterium* and *p-75-a5* can be highlighted. The strongest correlations found for CH_4 g/day were with Bifidobacteriaceae ($r = 0.62$, $P<0.05$) and the genera *Acetobacter* ($r = 0.81$, $P<0.01$), *Eubacterium* ($r = -0.76$, $P<0.01$) and *p-75-a5* ($r = -0.66$, $P<0.05$). Strongest correlations with CH_4 g/kg of DMI were with genera *Coprococcus* ($r = 0.61$, $P<0.05$), *Eubacterium* ($r = -0.76$, $P<0.01$*), p-75-a5* ($r = -0.66$, $P<0.05$). When analyzed by the relation with the digestible fraction of DMI and OMI, the most important correlations observed were with genera *Eubacterium* and *p-75-a5*.

Acetobacter, Bifidobacteriaceae, *Clostridium*, *Coprococcus, Fibrobacter* and *Schwartzia* showed the strongest positive correlations with dDMI and dOMI. The strongest negative correlations were observed with genera *Eubacterium* and *SHD-231*. For average body weight positive correlations were detected with *Acetobacter*, Bifidobacteriaceae, *Coprococcus* and RF16 and negative correlations with *Eubacterium* and *p-75-a5*.

Surprisingly, in the archaeal community, *Methanobrevibacter* was weakly correlated with CH_4 emissions. The strongest correlations were with CH_4 g/day and CH_4 g/kg of DMI ($r = 0.52$, $P<0.10$ and $r = 0.50$, $P<0.10$, respectively). When CH_4 emissions were analyzed in relation to dDMI and dOMI, the correlations were found to be even weaker. Genus *Methanosphaera* showed strong negative correlations with CH_4 g/day ($r = -0.85$, $P<0.01$), CH_4 g/kg of DMI ($r = -0.87$, $P<0.01$), CH_4 g/kg of dDMI ($r = -0.77$, $P<0.01$) and CH_4 g/kg of dOMI ($r = - 0.79$, $P<0.01$). Genus *Vadin CA11* was found to be positively correlated to CH_4 emissions, mainly when they were expressed in relation to dDMI and dOMI ($r = 0.66$, $P<0.01$ and $r = 0.65$, $P<0.05$, respectively). Only *Methanosphaera* significant correlated with dDMI ($r = -0.59$, $P<0.05$) and dOMI ($r = -0.60$, $P<0.05$). Body weight was correlated with *Methanosphaera* ($r = -0.88$, $P<0.01$) and *Vadin CA11* ($r = 0.53$, $P<0.10$).

Only genus *Orpinomyces* from anaerobic fungi community correlated with CH_4 emissions. The strongest correlation was observed with CH_4 g/kg of DMI ($r = 0.60$, $P<0.05$).

Surprisingly, none of the bacteria, archaea and anaerobic fungi which correlate with CH_4 emissions showed significant correlations ($P>0.1$) with VFA and the individual concentrations of acetate, propionate and butyrate.

3.8. Covariation among bacterial, archaeal and anaerobic fungal communities and between these communities and volatile fatty acids

Covariation among bacterial, archaeal and anaerobic fungi communities was accessed via Mantel's test. Neither bacterial and archaeal, bacterial and anaerobic fungal or archaeal and anaerobic fungal communities covaried ($P = 0.25$, $P = 0.98$ and $P = 0.98$ respectively). Volatile fatty acids and microbial communities did not covary either ($P = 0.53$ for bacteria, $P = 0.46$ for archaea and $P = 0.19$ for anaerobic fungi).

4. Discussion

In this study, it was shown that growing heifers raised in tropical conditions shared a core microbiome composed of *Prevotella*, *Ruminococcus*, *Coprococcus*, *Butyrivibrio*, *Clostridium*, *Shuttleworthia*, *SHD-231*, *CF231*, *p-75-a5, Methanobrevibacter, Methanosphaera,* and *Caecomyces communis*. Genus *Prevotella* is a relatively well-known genus, with four characterized rumen species, *P. ruminicola*, *P. bryantii*, *P. albensis* and *P. brevis* (Avgustin et al., 1997). Nevertheless, this genus shows a highly varied genetic divergence (Ramsak et al., 2000), which implies a possible great functional versatility, allowing the members of this genus to fill up in several ecological niches within the rumen (Jami and Mizrahi, 2012), which might explain its presence in core microbiome of several studies (Jami and Mizrahi, 2012; Petri et al., 2013; Kumar et al., 2015) regardless the type of diet. In forage based diets, Petri et al. (2013) and Kumar et al. (2015) also observed *Ruminococcus*, *Butyrivibrio*, *CF231*, *Methanobrevibacter*, and *Methanosphaera* as genera shared among all evaluated animals.

Samples of each heifer's physiological group or CH_4G did not cluster together in nMDS for archaea and anaerobic fungi, suggesting that there was not a clear distinction in structure of these groups in these communities. For the bacterial community of the heifers evaluated herein, we can observe a tendency of clustering for PP, PB and PG. In the present study, heifers were fed the same diet and the same silage quality throughout the experiment. Since forage quality was found to be the most important factor modulating the microbial community in tropical environment (Reis et al., 2016), the lack of a well-defined clustering for the physiological groups was not surprising. When considering the CH_4G and bacterial

community, we can observe a differentiation between high and low emitters and that all environmental variables contributed for the clustering of the samples.

Firmicutes (51.27%) and Bacteroidetes (26.44%) were the most abundant bacterial phyla noticed in this research, which is in agreement with previous studies in rumen microbiota (Cunha et al., 2011; Menezes et al., 2011; Lima et al., 2015). These most abundant phyla were followed by TM7 (11.67%), Proteobacteria (2.49%) and SR1 (2.36%). Relative abundances of Firmicutes did not differ between PP and others or between low and high emitters, results that are supported by Wallace et al. (2015) findings. Relative abundance of Bacteroidetes was similar for PP and PG and lower for PP when compared to PB. Interestingly, ratio Firmicutes:Bacteroidetes was higher for PP than others and for low than high emitters.

Besides being previously detected as part of the core microbiome of lactating cows (Jami and Mizrahi, 2012), phylum TM7 do not regularly appear in literature in abundances as high as it appeared in this study. In growing male and adult Hostein-Friesian cows (Lee at al., 2012) and goats (Cunha et al., 2011; Lee at al., 2012), this phylum is reported to contribute with less than 2% of the relative abundance. The concentrate:forage ratio used in those studies were 40:60, 70:30 and 90:10, respectively. One may say that these results were affected by the sampling method (rumen liquid phase only), however this difference might not be because of the liquid phase samples used in the present study, since Cunha et al. (2011) analyzed both liquid and solid ruminal phases. Kim et al. (2014) reported that phylum TM7 showed greater relative abundances (10.92%) on fecal microbiota from heifers fed silage/forage diet (70% corn silage and 30% alfalfa haylage) in comparison to a moderate and a high grain diet. Thus, considering that heifers in this study were fed 90% corn silage, it seems that the high abundance of this phylum might be explained by the more fibrous diet

or by the lower amount of starch in these diets, which are common features in production systems in tropical regions.

Genera affiliated sequences from archaeal community from growing heifers seem to follow the same pattern found in the literature for grown cattle and sheep (Wright et al., 2008; King et al., 2011; Kumar et al., 2015). The research of Wright et al. (2008) was also performed in a tropical region using the same ruminal sample collection method, so it appears that the geographical location does not influence the abundance of the archaeal community. The most abundant archaeal genus was *Methanobrevibacter* and lower contributions were from *Methanosphaera* and *Vadin CA11*.

In line with other studies (Liggenstoffer et al., 2010; Kumar et al., 2015), unclassified sequences corresponded to more than half of the anaerobic fungi sequences (52.23 %). They might represent novel ruminal taxa of anaerobic fungi and they are present in the dataset, probably, because of problems in assigning taxonomy rank to the sequenced reads (Kumar et al., 2015). Excepting the unclassified sequences, genus *Caecomyces* was the most abundant in heifers, as described previously by Liggenstoffer et al. (2010), which concluded that *Caecomyces* was the most abundant genus in data sets from domestic cattle. *Orpinomyces* abundance is also within the range (3% of the community composition) reported by Liggenstoffer et al. (2010).

Comparing the taxa present in this study and other research, surprisingly we observe that genus *Anaeromyces* (Liggenstoffer et al., 2010) and *Cyllamyces* (Ozkose et al., 2001; Kumar et al., 2015) are absent in the anaerobic fungal community of growing heifers in tropical environment. Apparently, these genera are usually present in the foregut of ruminants but their population establishment in rumen seems to be dependent on other factors, such as feed type and geographical location (Liggenstoffer et al., 2010). Taken together, these factors

summed to the different age and body weight of the heifers used in this study and the animals used in Ozkose et al. (2010) and Kumar et al. (2015) might explain the absence of these genera in the anaerobic fungal microbiome.

Diversity and richness (Shannon and Chao1 indexes) of bacterial, archaeal and anaerobic fungal communities were not different in heifers producing high or low CH_4. These indexes were not different among the archaeal community of PP, PB and PG heifers. Pubertal heifers had a more diverse bacterial community than PP heifers and a greater Chao1 index in anaerobic fungal community was found for PB in relation to PP heifers. Jami et al. (2013) showed an increase in bacterial communities' diversity with age, even in six months and two years old animals fed the same diet. According to these authors, there is a strategic direction toward a fully developed bacterial arrangement. Based on the present study, we suggest that growing animals might experience fluctuations in diversity and/or richness during different growing phases to reach the fully developed microbial arrangement above mentioned.

This study showed important correlations among some bacterial families and genera, and CH_4 emissions and the other production traits. Genus *Acetobacter*, which was strongly correlated with CH_4 emissions, dDMI, dOMI, and BW are acetic acid producers, mainly from ethanol, and belongs to phylum Proteobacteria. Little is known about *Acetobacter* in rumen, but Wallace et al. (2015) found Proteobacteria to be four fold more abundant in low emitters animals. In the present study, no differences in Proteobacteria phylum relative abundances were observed in the physiological groups or in CH_4G. But, genus *Acetobacter* was more abundant in PB, PG and high CH_4 emitters groups ($P<0.05$).

Bacteria from genera *Eubacterium* and *p-75-a5* followed the same pattern of inverse correlations with CH_4 g/day, CH_4 g/kg of DMI, CH_4 g/kg of dDMI and CH_4 g/kg of dOMI. Little is known about genera *Eubacterium* and *p-75-a5,* and their effects in lowering CH_4

emissions are probably driven by their negative influence on dDMI and dOMI. *Eubacterium* was previously reported as a non-saccharolytic ammonia-hyperproducing bacteria that plays important role in amino acids fermentation (Wallace et al., 2003). Genus *p-75-a5* is within family Erysipelotrichaceae and the role of this family in rumen fermentation is unknown (Menezes et al., 2011). These genera were more abundant in PP and low emitters heifers ($P<0.05$), which is supported by the negative correlation with BW.

Methanobrevibacter correlated weakly with CH_4 g/day ($r = 0.51$, $P<0.1$) and CH_4 g/kg of DMI ($r = 0.50$, $P<0.1$). Stronger correlation might be found in gene expression analysis of genes related to methanogenesis rather than relative abundances. *Methanobrevibacter* has been associated with higher CH_4 emissions by cattle and showed greater abundances in high CH_4 emitters animals (Wallace et al., 2015). Such result was obtained in samples from ruminal fluid phase and was confirmed in the present study, in which greater abundances of *Methanobrevibacter* were observed in groups that showed higher CH_4 emissions (PB, PG and high emitters heifers).

Methanosphaera exhibited strongly negative correlations with CH_4 emissions (< -0.74) and its abundance was higher in PP and low CH_4 emitters heifers ($P<0.01$). Such finding is consistent with Kittelmann et al. (2014) results, in which the authors also found genus *Methanosphaera* to be significantly more abundant in rumen fluid of low-methane emitters sheep. However, Wallace et al. (2015) detected a greater abundance of *Methanosphaera* in high CH_4 emitters beef cattle. In the present study, PP animals exhibited the highest *Methanosphaera* abundance and the lowest CH_4 emission. Besides, when grouped in terms of CH_4 emissions, the low emitters group had highest *Methanosphaera* abundance ($P<0.01$).

Little is known about genus *Vadin CA11* but it showed significant correlations with CH_4 emissions, especially when it was expressed in relation to dDMI and dOMI. This genus relative abundance did not differ between high and low emitters, but it was present in a greater abundance in PG heifers ($P<0.10$).

Only genus *Orpinomyces* was found to correlate with CH_4 emissions, being the strongest correlation with CH_4 g/kg of DMI ($r = 0.60$, $P<0.05$). These correlations were expected, since anaerobic fungi are responsible for colonizing fibrous materials, making it more easily degradable, thus producing more acetate and, therefore, more CH_4. Also, because of the role of anaerobic fungi in fiber degradation, correlations between anaerobic fungi and dDMI and dOMI were expected, but *Orpinomyces* showed weak and non-significant correlations ($r = 0.41$, $P>0.10$) with the digestible fractions of feeds.

In summary, PP and low emitters heiferes showed the lower values for CH_4 g/kg dOMI, and lower CH_4 emission in g/kg dDMI was found for PP than PG. These differences in CH_4 emissions can be seen as different forms that microbiota interacts and works on the substrate. Given that acetate, propionate and butyrate concentrations in samples from PP and PG were similar ($P>0.10$), and that PP showed lower dDMI and dOMI, we can conclude that predominant pathways inside rumen of each group were different and that PP heifers could be more efficient in degradation process than PG, due to characteristics of their microbiota. Pathways characteristics can be accessed by the expression of genes related to methanogenesis, which is a suggestion for further studies.

In the archaeal community, *Methanobrevibacter* and *Vadin CA11* were positively correlated with CH_4 and its relative abundances were lower for PP than PG. In fact, 30% of the differences among PP and PG communities were because of *Methanobrevibacter* (Table 3). On the other hand, *Methanosphaera* showed strong negative correlations with CH_4

emissions, its relative abundances were lower for PP in comparison with PG and 22 and 27% of the differences among these communities were due to OTUs affiliated as *Methanosphaera*.

In general, only few differences were detected in this study that might be attributed to the tropical environment. Forage quality is one of the greatest challenges in tropical systems, but it seems that quality of the silage used in the present study was not a divergent point with the temperate systems. Despite all data generated herein, further research is required to confirm some results obtained in this study and to better understand of the associations described here.

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6. References

Anderson, K. L., T. G. Nagaraja, J. L. Morrill, T. B. Avery, S. J., Galitzer, and J. E. Boyer. (1987). Ruminal microbial development in conventional or early weaned calves. J. Anim. Sci. 64:1215–1226.

Association of Official Analytical Chemists (AOAC) 1990 Official Methods of Analyses of the AOAC, pp. 1 –1230, 15 edition. Washington: Assoc OFF Agric Chemp.

Avgustin, G., Wallace, R.J., Flint, H. (1997). 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: 284–288.

Beharka, A.A., Nagaraja, T.G., Morrill, J.L., Kennedy, G.A., Klemm, R.D. (1998). Effects of form of the diet on anatomical, microbial, and fermentative development of the rumen of neonatal calves. J Dairy Sci 81:1946–1955.

Charrad, M., Ghazzali, N., Boiteau, V., Niknafs, A. (2014). NbClust: An R Package for Determining the Relevant Number of Clusters in a Data Set. Journal of Statistical Software, 61(6), 1-36. URL http://www.jstatsoft.org/v61/i06/.

Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18:117–143.

Clarke K.R., Warwick, R.M. (1994). Change in marine communities: an approach to statistical analysis and interpretation. Natural Environment Research Council, UK.

Cunha, I.S., Barreto, C.C., Costa, O.Y.A., Bomfim, M.A., Castro, A.P, Kruger, R.H., Quirino, B.F. (2011). Bacteria and Archaea community structure in the rumen microbiome of goats (Capra hircus) from the semiarid region of Brazil. Anaerobe, 17:118-124.

Detmann, E., Souza, M.A., Valadares Filho, S.C., Queiroz, A.C., Berchielli, T.T., Saliba, E.O.S., Cabral, L.S., Pina, D.S., Ladeira, M.M., Azevêdo, J.A.G. (2012). Métodos para análise de alimentos, first ed. Suprema, Visconde do Rio Branco, MG, Brazil.

Henderson, G., Cox, F., Kittelmann, S., Miri, V.H., Zethof, M., Noel, S.J., Waghorn, G.C., Janssen, P.H. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. Plos One 597 8:e74787.

Intergovernmental Panel on Climate Change. Climate change 2014. Synthesis report 2014.

Jami, E., Mizrahi, I. (2012). Composition and similarity of bovine rumen microbiota across individual animals. PLoS ONE 7(3): e33306.

Jami, E., Israel, A., Kotser, A., Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7 (6), 1069 – 1079.

Jiao, H. P., T. Yan, D. A. Wills, A. F. Carson, and D. A. McDowell. (2014). Development of prediction models for quantification of total methane emission from enteric fermentation of young Holstein cattle at various ages. Agric. Ecosyst. Environ. 183:160–166.

Johnson, K.A., Huyler, M., Westberg, H.H., Lamb, B. and Zimmerman, P. (1994). Measurement of methane emissions from ruminant livestock using a sulphur hexafluoride tracer technique. Environ. Sci. Technol. 28:359–362.

Johnson, K.A., Johnson, D.E. (1995). Methane emissions from cattle. Journal of Animal Science. 73:2483-2492.

Johnson, K.A., Westberg, H. H., Michal, J. J. and Cossalman, M.W. (2007). The SF_6 tracer technique: Methane measurement from ruminants. In: Makkar H. S. and Vercoe P. (eds.) Measuring methane production from ruminants, pp. 33-67: Springer Netherlands.

Kim, M., Kim, J., Kuehn, L.A., Bono, J.L., Berry, E.D, Kalchayanand, N., Freetly, H.C., Benson, A.K., Wells, J.E. (2014). Investigation of bacterial diversity in the feces of cattle fed different diets. J. Anim. Sci.92:683–694.

King, E.E., Smith, R.P., St-Pierre, B., Wright, A.D.G. (2011). Differences in the rumen methanogen populations of lactating Jersey and Holstein dairy cows under the same diet regimen. Appl Environ Microbiol.77: 5682–5687.

Kittelmann, S., Seedorf, H., Walters, W.A., Clemente, J.C., Knight, R., Gordon, J.I., Janssen, P. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. PLoS ONE 8(2): p. e47879.

Kittelmann, S., Pinares-Patiño, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., McEwan, J.C., Janssen, P. (2014) Two Different Bacterial Community Types Are Linked with the Low-Methane Emission Trait in Sheep. PLoS ONE 9(7): e103171.

Kumar, S., Indugu N., Vecchiarelli, B., Pitta, D.W. (2015). Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to

changes in diet and age in the rumen of dairy cows. Front. Microbiol. 6:781.

Kurihara, M., Magner, T., Hunter, R. A., McCrabb, G. J. (1999). Methane production and energy partition of cattle in the tropics. Br. J. Nutr. 81, n.3, p.227-234.

Lee, H.J., Jung, J.Y., Oh, Y.K., Lee, S.S., Madsen, E., Jeon, C.O. (2012). Comparative Survey of Rumen Microbial Communities and Metabolites across One Caprine and Three Bovine Groups, Using BarCoded Pyrosequencing and 1H Nuclear Magnetic Resonance Spectroscopy. Appl Environ Microbiol. 78: 5983–5993.

Liggenstoffer, A. S., Youssef, N. H., Couger, M., Elshahed, M. S. (2010). Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. ISME J. 4: 1225–1235.

Lima, F.S., Oikonomou, G., Lima, S.F., Bicalho, M.L.S., Ganda, E.K., Oliveira Filho, J.C., Lorenzo, G., Trojacanec, P., Bicalho, R.C. (2015). Prepartum and postpartum rumen fluid microbiomes: characterization and correlation with production traits in dairy cows. Appl Environ Microbiol 81:1327–1337.

Lodge-Ivey, S.L., Browne-Silva, J., Horvath, M.B. (2009). Technical note: Bacterial diversity and fermentation end products in rumen fluid samples collected via oral lavage or rumen cannula. J. Anim. Sci. 87:2333–2337.

Mantel N. (1967). The detection of disease clustering and a generalized regression approach. Cancer Res. 27:209–220.

Mendiburu, F. (2016). Agricolae: Statistical Procedures for Agricultural Research. R package version 1.2-4. http://CRAN.R-project.org/package=agricolae.

Menezes, A.B., Lewis, E., O'Donovan, M., O'Neill, B.F., Clipson, N., Doyle, E.M. (2011). Microbiome analysis of dairy cows fed pasture or total mixed ration diets. FEMS Microbiol. Ecol.78, 256–265.

Ozkose, E., Thomas, B.J., Davies, D.R., Griffith, G.W., Theodorou, M.K. (2001). Cyllamyces aberensis gen. nov. sp. nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. Can J

Bot. 79: 666–673.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H. (2015). Vegan: community ecology package. URL http://vegan.r-forge.r-project.org/.

Petri, R.M., Schwaiger, T., Penner, G.B., Beauchemin, K.A., Forster, R.J., Mckinnon, J.J., McAllister, T.A. (2013) Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PLoS ONE 8(12): e83424.

Primavesi, O., Frighetto, R.T.S., Pedreira, M.S., Lima, M.A., Berchielli, T.T., Demarchi, J.J.A.A., Manella, M.Q., Barbosa, P.F., Johnson, K.A. and Westberg, H.H. (2004). Técnica do gás traçador sf6 para medição de campo do metano ruminal em bovinos: Adaptações para o Brasil, p. 76. São Carlos - SP: Embrapa.

R Core Team (2015) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

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

Reis, W.L.S., Detmann, E., Batista, E.D., Rufino, L.M.A., Gomes, D.I., Bento, C.B.P., Mantovani, H.C., Valadares Filho, S.C. (2016). Effects of ruminal and post-ruminal protein supplementation in cattle fed tropical forages on insoluble fiber degradation, activity of fibrolytic enzymes, and the ruminal microbial community profile. Animal Feed Science and Technology. 218, 1-16.

Siegfried, B. R.; Ruckemann, H.; Stumpf, G. (1984). Method for the determination of organic acids in silage by high performance liquid chromatography. Landwirtsch. Forsch, v.37, p.298.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F. (2009) Introducing mothur: open-source, platform-independent, community supported software for describing and

comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.

Stevenson, D., and Weimer, P. (2007) 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: 165–174.

Stewart, C.S., Flynt, H.J., Bryant, M.P. (1997). The rumen bacteria, p. 10–72. In Hobson PN, Stewart CS (ed), The rumen microbial ecosystem. Blackie Academic and Professional, New York, NY.

Wallace, R. J., N. McKain, N. R. McEwan, A. E. Miyagaw, L. C. Chaudhary, T. P. King, N. D. Walker, J. H. Apajalahti, and C. J. Newbold. (2003). Eubacterium pyruvativorans sp. nov., a novel non-saccharolytic anaerobe from the rumen that ferments pyruvate and aminoacids, forms caproate and utilizes acetate and propionate. Int. J. Syst. Evol. Microbiol. 53:965–970.

Wallace, R.J., Rooke, J.A., McKain, N., Duthie, C.A., Hyslop, J.J., Ross, D.W., Waterhouse, A., Watson, M., Roehe, R. (2015). The rumen microbial metagenome associated with high methane production in cattle. BMC Genomics. 16:839.

Wei, T. (2012) Package 'Corrplot' – Visualization of a correlation matrix v0.60. cran.rproject.org. URL http://cran.r-project.org/web/packages/corrplot/corrplot.pdf.

Wright, A.D.G., Williams, A.J., Winder, B., Christophersen, C.T., Rodgers, S.L., Smith, K.D. (2004). Molecular diversity of rumen methanogens from sheep in Western Australia. Applied and Environmental Microbiology. 70:3, 1263-1270.

Wright, A.D.G., Ma, X., Obispo, N.E. (2008). Methanobrevibacter phylotypes are the dominant methanogens in sheep from Venezuela. Microb. Ecol. 56:390–394.

Table 1. Percentage of ingredients and chemical composition of concentrate and experimental diet

Item	Concentrate	Diet
Corn silage		90.00
Corn meal	39.30	3.93
Soybean meal	44.20	4.42
Urea	12.70	1.27
Ammonium sulfate	3.40	0.34
Mineral mix	0.50	0.05
Nutritional composition		
Dry matter	90.26	39.65
Crude protein	68.58	13.98
Ether extract	2.38	3.06
Neutral detergent fiber	11.87	51.46
Mineral mix	4.45	4.95

Table 2. Methane emissions and production traits of growing heifers

Animal	CH_4 (g/ day)	CH_4 (g/kg DMI)	CH_4 (g/kg dDMI)	CH_4 (g/kg dOMI)	dDMI (kg/day)	dOMI (kg/day)	BW (kg)	DMI/kg of BW	Total VFA (mmol/L)	Acetate (mmol/L)	Propionate (mmol/L)	Butyrate (mmol/L)	A:P
PP1	97.94	18.52	27.59	28.62	3.55	3.42	244.50	0.022	92.06	58.62	19.72	9.03	2.97
PP2	69.74	15.36	19.32	20.34	3.61	3.43	230	0.020	94.16	59.50	19.57	9.51	3.04
PP3	94.17	19.52	29.61	30.60	3.18	3.08	208	0.023	90.80	57.06	20.11	8.27	2.84
PP4	92.39	21.68	32.78	33.77	2.82	2.74	182.50	0.023	83.05	54.76	16.08	8.97	3.41
PP5	62.90	17.61	27.61	28.20	2.28	2.23	155	0.023	99.38	65.51	20.25	8.77	3.23
Means	83.43C	18.54C	27.38B	28.30B	3.09B	2.98B	204C	0.022A	91.89A	59.09AB	19.15A	8.91A	3.10B
PB1	196.87	27.87	39.64	41.10	4.97	4.79	351.50	0.020	80.48	54.84	16.68	6.42	3.29
PB2	215.53	25.66	32.59	34.32	6.61	6.28	393	0.021	87.48	58.80	16.70	7.14	3.52
PB3	120.02	22.87	42.72	42.95	2.81	2.79	329	0.016	73.21	49.53	14.83	6.07	3.34
PB4	161.95	21.93	28.33	30.03	5.72	5.39	360.50	0.020	76.98	53.28	15.98	6.05	3.33
Means	173.59B	24.58B	35.82AB	37.10A	5.03A	4.81A	358.5B	0.019B	79.54B	54.12B	16.05B	6.42B	3.37A
PG1	229.58	31.54	41.75	43.25	5.50	5.31	515	0.014	101.00	67.44	19.56	10.10	3.45
PG2	209.44	31.18	44.20	45.74	4.74	4.58	420	0.016	109.74	72.75	22.41	10.00	3.25
PG3	198.81	26.43	37.61	38.81	5.29	5.12	464	0.016	99.21	66.90	18.98	8.81	3.53
PG4	229.27	29.48	41.58	42.95	5.51	5.34	539	0.014	97.39	65.24	19.77	7.81	3.30
PG5	215.27	36.51	54.06	54.73	3.98	3.93	427	0.014	83.75	55.64	16.41	7.29	3.39
Means	216.47A	31.03A	43.84A	45.10A	5.00A	4.86A	473A	0.015C	98.22A	65.59A	19.42A	8.80A	3.38A

CH_4= methane; dDMI= digestible dry matter intake; dOMI= digestible organic matter intake; BW= average body weigth; DMI= dry matter intake; VFA= volatile fatty acid (acetate, propionate, butyrate, lactate, isobutyrate, isovalerate, valerate); A:C= acetate:propionate ratio. PP= prepubertal heifers; PB= pubertal heifers; PG= pregnant heifers. Means followed by different letters in a column are different by Tukey test ($P<0.10$).

Table 3. Percentages of influence of the OTU in differences in archaeal community of growing heifers' groups (PP, PB and PG) and CH_4 emission level groups (high and low)

OTU #	OTU0001	OTU0002	OTU0003	OTU0006
Archaea genera	*Methanobrevibacter*	*Methanosphaera*	*Methanosphaera*	*VadinCA11*
PP x PB	0.35	0.31	0.26	-
PP x PG	0.30	0.27	0.22	-
PB x PG	0.34	-	0.16	0.25
High CH_4 x Low CH_4	0.31	0.27	0.24	-

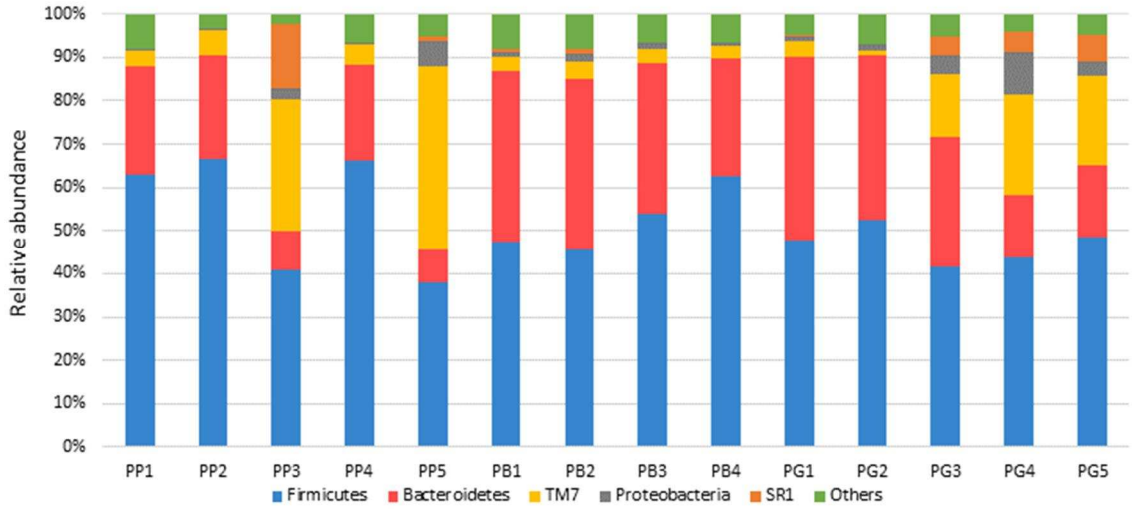

Figure 1. Relative abundance of bacterial community of growing heifers at phylum level. PP= prepubertal heifers; PB= pubertal heifers; PG= pregnant heifers.

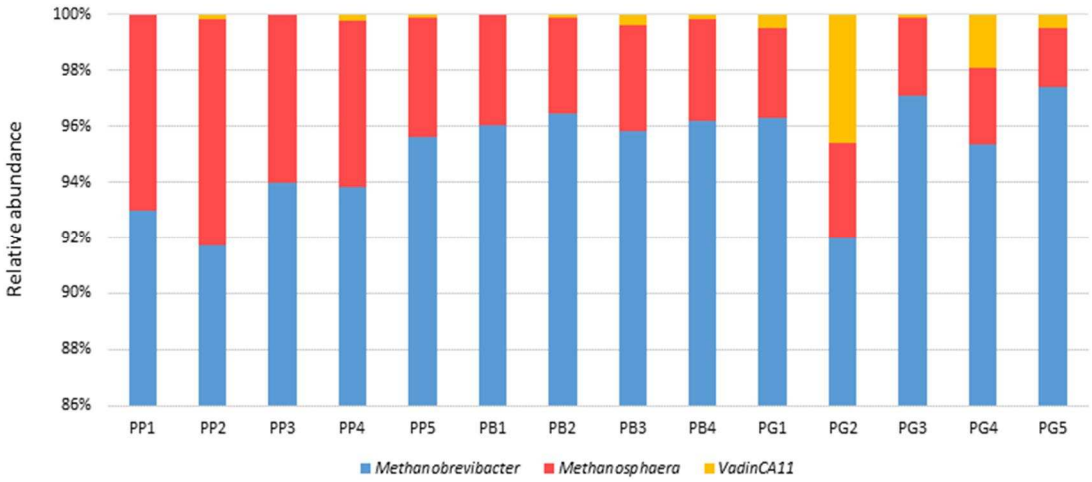

Figure 2. Relative abundance of archaeal community of growing heifers at genus level. PP= prepubertal heifers; PB= pubertal heifers; PG= pregnant heifers.

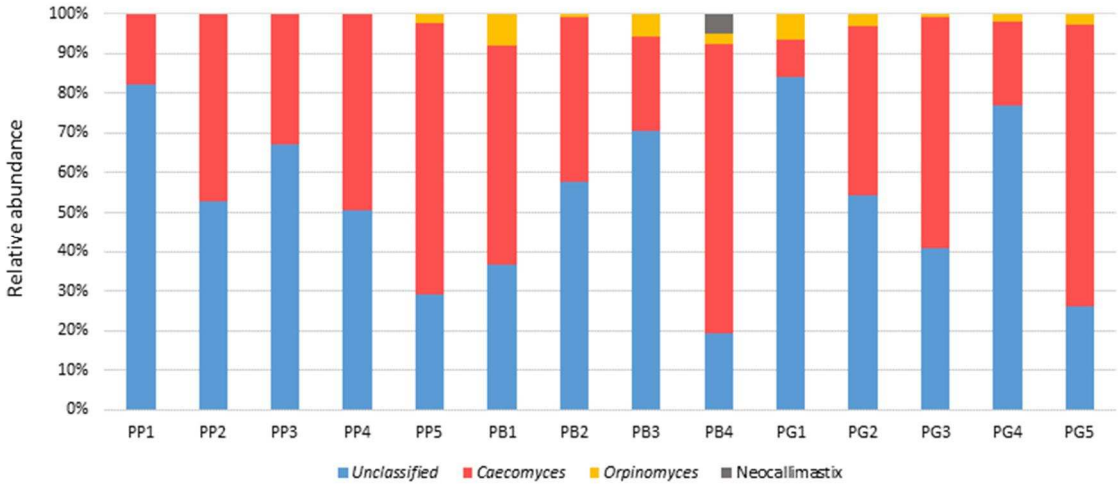

Figure 3. Relative abundance of anaerobic fungal community of growing heifers at genus level. PP= prepubertal heifers; PB= pubertal heifers; PG= pregnant heifers.

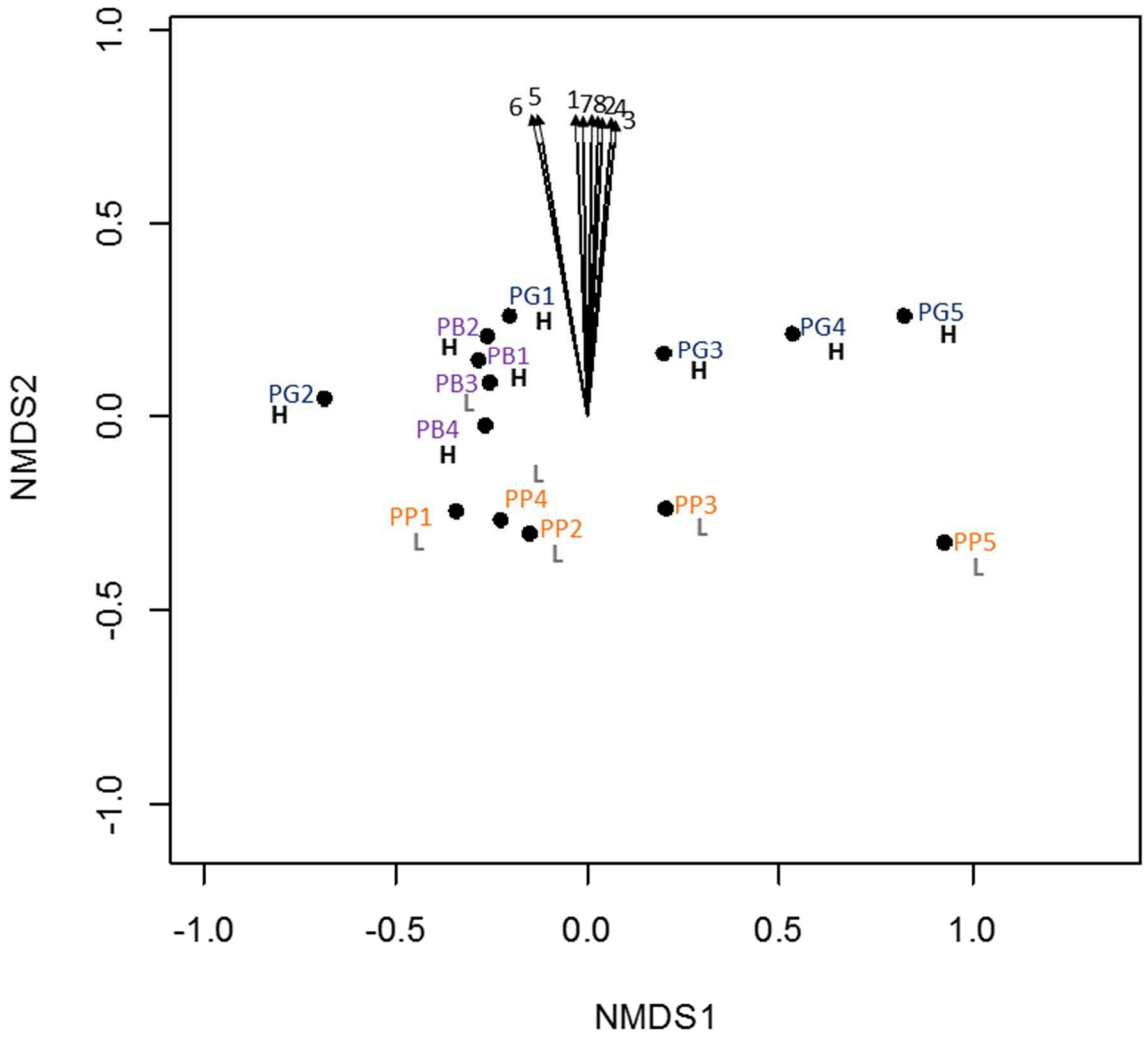

Figure 4. Non-metric multidimensional scaling plot of Bray-Curtis dissimilarity index of bacterial community structure. 1= CH_4 g/day; 2= CH_4 g/kg DMI; 3= CH_4 g/kg dDMI; 4= CH_4 g/kg dOMI; 5= dDMI; 6= dOMI; 7= BW; 8= acetate:propionate ratio. PP= prepubertal heifers; PB= pubertal heifers; PG= pregnant heifers; L= low methane emitters; H= high methane emitters. Each data point represents the microbial community of each evaluated heifer.

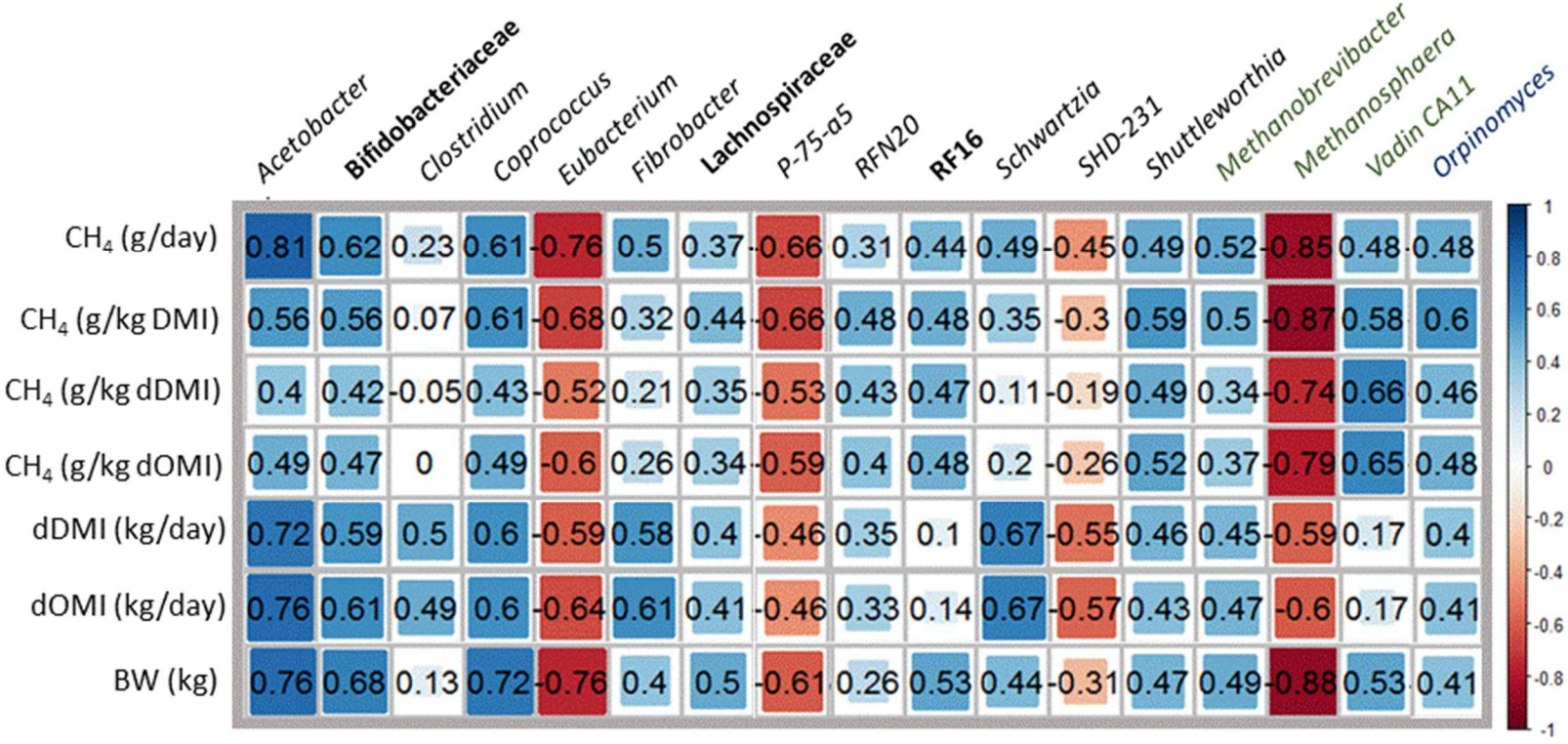

Figure 5. Correlation between CH_4 emissions and other production parameters and relative abundance at family and genus taxonomic level. Spearman correlation matrix of selected family/genus across all samples. Names in bold means family, the other refers to genus taxonomic level. Names in black, green and blue means, respectively, bacteria, archaea and anaerobic fungi.

CHAPTER 2

METHANE EMISSIONS AND PRODUCTION TRAITS OF HOLSTEIN COWS IN LACTATION OR NOT AND THEIR RELATIONSHIPS WITH BACTERIAL, ARCHAEAL AND ANAEROBIC FUNGAL RUMEN COMMUNITIES

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Abstract

The aim of this study was to characterize rumen bacterial, archaeal and anaerobic fungal communities, through sequencing of 16s rRNA and ITS genes, of high-producing (HP), medium-producing (MP), low-producing (LP) and dry (DC) cows in a tropical production system. Moreover, we accessed the relationship between these communities and enteric methane (CH_4) emissions and productive traits, such as digestible dry matter intake (dDMI), digestible organic matter intake (dOMI), volatile fatty acids (VFA) and its main components, acetate, propionate and butyrate. The forage:concentrate ratios were 50:50 for HP, 70:30 for MP, 80:20 for LP, and 90:10 for DC. Considering the intake of digestible fraction of feed, DC emitted more CH_4, followed by MP, HP and LP, but the HP and LP emissions were similar. The core microbiome of Holstein cows in tropical environment, irrespective to their production and the diet they were fed, is composed by *Prevotella*, *Ruminococcus*, *Butyrivibrio*, *Clostridium*, *Coprococcus*, *Shuttleworthia*, *CF231*, *SHD-231*, *Methanobrevibacter*, and *Methanosphaera*. None of the anaerobic fungal operational taxonomic units (OTU) were found in all rumen fluid samples. Firmicutes and Bacteroidetes were the most abundant phyla found in the rumen of Holstein cows. For the archaeal community, *Methanobrevibacter* genera was the most abundant and in anaerobic fungi, most of the sequences were unclassified. The strongest negative correlations with CH_4 emissions were with the relative abundance of family Coriobacteriaceae and S24-7 and of genera *Butyrivibrio, Clostridium* and *Schwartzia*. Positive correlations were found between CH_4 emissions and families RF16 and Succinivibrionaceae. In the archaeal community, genera *Methanosphaera* relative abundance showed a negative correlation with CH_4. Surprisingly, no significant correlation between CH_4 emissions and *Methanobrevibacter* relative

abundance was found. Relative abundance of genera *Vadin CA11* (archaea) and *Caecomyces* (anaerobic fungi) were detected to be positively correlated with CH_4 in g/day. Many families and genera from Firmicutes phylum showed positive correlations with dDMI and dOMI. None of the bacterial, archaea and anaerobic fungi which correlate with CH_4 emissions showed significant correlations (P>0.1) with VFA and the individual concentrations of acetate, propionate and butyrate. The most opposite results observed in the present study were among DC and HP. Dry cows showed greater CH_4 emissions in g/kg dDMI and g/kg of dOMI and, besides no differences were observed in relative abundances of Firmicutes, Bacteroidetes and Firmicutes:Bacteroidetes ratio. Dry cows had lower relative abundance of Coriobacteriaceae, which was negatively correlated with CH_4, and greater relative abundance of Succinivibrionaceae, that was positively correlated with CH_4. In addition, DC had greater relative abundance of *Methanobrevibacter* and lower of *Methanosphaera*. Lastly, bacterial, archaeal and anaerobic fungal communities did not covary and VFA and microbial communities did not vary in a similar way either.

1. Introduction

The rumen is inhabited by microorganisms such as bacteria, archaea, anaerobic fungi and protozoa. They interact with each other to coordinate the fermentation processes of different feed components. During degradation of feed components, most of methanogens (archaea) use hydrogen (H_2) and carbon dioxide (CO_2) released by other microorganisms to produce methane (CH_4) (Stewart et al., 1997). Thus, the microbiota that the animal harbors in its rumen is the responsible for producing volatile fatty acids used as energy for growth, reproduction and to produce milk and meat.

It has been demonstrated that high milk producing cows emit less CH_4 per unit of intake and per litter of milk produced, in comparison to low milk producing cows (Yan et al., 2010). This fact is explained by a dilution of the maintenance requirements, due to an increase in energetic efficiency of the cows (Bauman et al., 1985; Yan et al., 2010; Connor et al., 2012). However, few information on CH_4 emissions of cows with different energetic efficiencies, and therefore, distinct levels of milk production, in the same production system are found in literature (Yan et al., 2010).

Factors such as type of feed (Menezes et al., 2011) and quality of forage (Reis et al., 2016) that animals consume are keys in modulating the availability of H_2 to be used by methanogens. A more fibrous diet leads to more residual H_2 because of the greater amounts of acetate produced, while diets based on high starch feeds induce a greater production of propionate, lowering the production of H_2, thus lowering CH_4 production. These factors are strictly bound to climate and geographical conditions in which the production system is located, since in tropical conditions forages often present lower digestibility and their degradation in rumen results in a higher proportion of acetate. These peculiarities suggest a need for studies that can show evidence of the divergent points between different climates and geographical conditions.

Since degradation and methanogenesis in rumen are dependent of the complexity of its microorganisms, we hypothesize that differences in bacterial, archaeal and fungal communities composition might help in explaining the differences seen in CH_4 emissions of Holstein cows from different levels of milk production. Thus, this study aimed to characterize the rumen microbiota of lactating and dry cows, under different diets, in a tropical system of production, and to investigate the relationship between ruminal microbial community and enteric CH_4 production and other production traits.

2. Material and methods

2.1. Animals and management

The experiment was conducted at the Unit for Teaching, Research, and Extension on Dairy Cattle (UEPE-GL) of the Universidade Federal de Viçosa (UFV), Brazil and at the Microbial Sciences Department at University of Wisconsin, USA. The project was approved by the Ethics Committee in the Use of Production Animals from the UFV (protocol number 99/2014).

In a functioning dairy system at UEPE-GL, five dry cows (DC) and 14 cows were selected. Lactating cows were split according to their production level, in high-producing cows (HP, 25±2.74 L milk/d; n = 5); medium-producing cows (MP, 15±0.85 L milk/d; n = 5) and low-producing cows (LP, 10±0.76 L milk/d; n = 4). All cows were housed in individual tie stalls and kept under the feeding management to which they were already adapted in the production system. Cows in lactation were automatically milked twice a day, at 6 am and 3 pm.

Each group trial was carried out separately. From day one to day five the animals were adapted to tie stall housing and to yokes. Gas collections from animals' eructation for evaluation of CH_4 emissions began on the sixth day. These collections started at 7 am through 24 hours and were performed, at least, until the 10^{th} day. Since the experiment was designed to obtain five high quality gases samples of each animal, in case of problems in the sampling process, the collection of that day was repeated after day 10. Evaluation of voluntary intake and milk production records were performed from day 6 to 10, feces samples were taken in day 6 and rumen samples, in day 10.

The diet supplied to the cows consisted of corn silage and concentrate (Table 1). High-

producing cows also received Coastcross grass (*Cynodon sp.*) hay in the proportion of 8.5% of total DM (41.5% of corn silage). All concentrates were based on corn, soybean meal and minerals. The forage:concentrate ratios were 50:50 for high-producing cows, 70:30 for the medium-producing cows, 80:20 for low-producing cows, and 90:10 for dry cows. Animals were fed twice a day, at 7 am and 3 pm and water was available *ad libitum* to all animals. At the beginning and end of the trials the animals were weighted before feeding and after milking without fasting.

2.2. Food, orts and feces sampling

Through five days, starting at day 6 in each trial, forage and orts from each animal were sampled. The quantities of food provided, and the weight of the orts were registered. Concentrate ingredients were sampled during mixing. At day 6, feces were collected during a 24 hour period. After this period, samples were taken. Food, orts and feces samples of each animal were grouped per trial, and were analyzed for dry matter (DM; Detmann et al., 2012; method INCT-CA nº G-003/1), crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), non-fiber carbohydrates (NFC) and ashes (MM) contents (AOAC, 1990) and the results were used for the calculation of the dry matter intake (DMI), dry matter digestibility (DMD), organic matter intake (OMI) and organic matter digestibility (OMD).

2.3. Methane emissions measurements

Animals' methane emissions measurements were performed by the tracer gas sulfur hexafluoride (SF_6) technique (Johnson et al., 1994) including adaptations proposed by Primavesi et al. (2004). Briefly, the flow of CH_4 released by the animal was calculated in relation to the flow of SF_6, measured from the SF_6 release rate from the permeation capsule

lodged in the rumen and from the concentrations of CH_4 and SF_6 in gas samples (Johnson et al., 2007). On the 4th day of each trial, the animals were forced to ingest one permeation capsule with known SF_6 release rate. To collect the gases, a PVC-tube collection-container yoke was used with a halter coupled to a collecting apparatus. Before the beginning of each collection day, the yokes went through a cleaning process, were evacuated at approximately –14 PSI, and then placed on the neck of each animal and coupled to the halter using a quick attachment. After 24 h, the yokes that were on the animals were switched for a cleaned and evacuated yokes, and the pressure of each yoke was recorded. The yokes containing samples of gases were pressurized with approximately 14 PSI of N_2 gas 99.999% purity. After a period of at least 1 h for homogenization of gases inside the yoke, the pressure was recorded once again, and samples were collected in previously evacuated Exetainer® tubes.

Analysis of CH_4 and SF_6 levels were performed at the Laboratory of Gas Chromatography of EMBRAPA Dairy Cattle, in Juiz de Fora, Minas Gerais, Brazil. The concentrations of CH_4 and SF_6 in the samples were evaluated in gas chromatograph. For SF_6 analysis, the detector μECD was used. The sampler system was bounding a 0.5 mL loop to a valve. In let typo split-splitless was used in splitless mode at 250 ºC. The separation system was composed by a column Molesieve 30 m × 0.530 mm × 25.0 μm, using gas N_2 as carrier gas at 5.0 mL/min in the column. Certified standards were used in the following concentrations for calibration of the chromatograph: 30, 100, 500; 1500 and 3000 ppt. For CH_4 analysis, a chromatograph equipped with two 6-way valves was used. One of the valves was used for the sampler system and the other one was used as a selector, allowing or not the passage of the constituents through the second column. In let typo split-splitless was used in splitless mode at 120 ºC. Separation system was composed by two columns (HP-Plot/Q 30m × 0.530 mm × 40.0 m and HP-Molesieve 30 m × 0.530 mm × 25.0 μm), using H_2 as carrier

gas at a flow rate of 7.0 mL/min. Detector TCD and FID were used. The calibration was performed using certified standards in the concentrations 4.8, 9.7, 19.6, 102 and 203 ppm. The results were expressed in g of CH_4/animal/day, g of CH_4/kg of DMI, g of CH_4/kg of digestible DMI (dDMI) and g of CH_4/kg of digestible OMI (dOMI).

2.4. Ruminal sampling and fermentation pattern

Ruminal content was sampled four hours after the feeding time using the stomach tube technique (Lodge-Ivey et al., 2009; Henderson et al., 2013; Kumar et al., 2015). The initial 200 mL of collected fluid were discarded, to avoid saliva contamination. Because of the collection method, rumen samples represented the liquid phase of ruminal content. A sample of approximately 70 mL was filtered through four layers of cheesecloth and split in two aliquots. The first one was destined for DNA extraction (50 mL), and the other one (20 mL), for volatile fatty acids (VFA) concentration analysis. Both samples were frozen at -80ºC until the time they were analyzed.

Volatile fatty acids (VFA) concentrations were analyzed using high-performance liquid chromatography (HPLC). Briefly, after a centrifugation step, 1.5 mL of a cell-free supernatant was treated as described by Siegfried et al. (1984) and was analyzed in a chromatograph Dionex Ultimate 3000 Dual with a refractive index detector Shodex RI-101 at 45 ºC, using an ion-exclusion column Phenomenex Rezex ROA, 300 x 7.8 mm, also at 45 ºC. The mobile phase was H_2SO_4 (5 mM) at a flow rate of 0.7 mL/min.

2.5. DNA extraction, amplicon library preparation and sequencing

DNA extractions were performed as described by Stevenson & Weimer (2007) and then treated with RNAse Ribonuclease A (10 mg/mL). Total DNA was quantified using a Qubit® 2.0 Fluoremeter (Invitrogen, San Diego, CA, USA). Two Polymerase Chain Reactions (PCR) were perfomed. The primers used aimed V3 and V4 regions of the 16S ribosomal RNA gene (16S rRNA) for Bacteria, the regions V6 and V8 of the same gene for Achaea, and the ITS1 gene for Fungi (Kittelmann et al., 2013).

The first PCR aimed to amplify region of interest using specific primers with Illumina specific overhand adapters attached and the second one to attach dual indices and Illumina sequencing adapters. The reactions were made under the following conditions: 95º C for three minutes, 25 cycles of 95 ºC for 30 seconds, 55 ºC for 30 seconds, 72 ºC for 30 seconds and 72 ºC for five minutes for the first PCR reaction and 95 ºC for three minutes, eight cycles of 95 ºC for 30 seconds, 55 ºC for 30 seconds, 72 ºC for 30 seconds and 72 ºC for 5 minutes for the second PCR reaction. This PCR were performed using 5 - 200 ng DNA, 0.5 µL of each primer (forward and reverse), master mix Kapa Biosystems® (Wilmington, MA, United States) and ultrapure water, totalizing a 25 µL reaction. Between both PCR reactions, a clean-up step was performed using an Invitrogen Pure Link Pro96 PCR Purification Kit. The products of the second PCR were loaded in a AquaPor LM low-melt agarose and the gel extraction was performed using the ZR-96 Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). DNA content of each sample was then quantified using a Qubit® 2.0 Fluoremeter, diluted, and pooled to 2 nM concentration solution. The library was denaturated at 96 ºC using NaOH 0.2 N, then diluted with Hybridization Buffer to 20 pM, and combined with the PhiX control library (20 pM). Then, the library was loaded in the Illumina MiSeq cartridge and the sequencing run at Illumina MiSeq 2x300bp was performed as indicated at the manufacturer's guidelines.

2.6. DNA sequence processing analysis

The programme MOTHUR, version 1.36.1 (Schloss et al., 2009), was used to analyze the obtained libraries. Sequences that were not aligned, chimera sequences and all contaminants were removed. The sequences from Bacteria and Archaea were aligned against the SILVA 16S rRNA gene database and classified using the Greengenes database. For anaerobic fungal analysis, sequences were classified using the Unit database and contaminants were removed as well. Sequence similarities greater than 97% were used as cut-offs to classify OTU at genus level. All sequences used for this study were publicly deposited in the NCBI Short Read Archive.

2.7.Microbial communities' analysis

Relative abundances were calculated as the number of sequences of the taxa divided by the number of total sequences present in the sample times 100. Alpha diversity indexes for richness and diversity, Chao 1 and Shannon, and Good's Coverage were calculated in MOTHUR version 1.35.0 (Schloss et al., 2009).

2.8. Statistical analysis

In R programme (R Core Team, 2015), Vegan package (Oksanen et al., 2015) was used to test differences in CH_4 emissions from each group using ANOVA, followed by a Tukey test, when necessary. Vegan package (Oksanen et al., 2015) was used to test differences in richness and diversity of bacterial, archaeal and anaerobic fungal communities.

The non-parametric test, Kruskal-Wallis (Agricolae package; Mendiburu, 2016) was used to test the differences in relative abundances. For these tests, $P<0.10$ were accepted as different.

Bacterial, archaeal and anaerobic fungal communities were visualized using a non-metric multidimensional scaling (nMDS) plots of the Bray-Curtis dissimilarity index. Non-metric multidimensional scaling was performed using Vegan package (Oksanen et al., 2015).

Analysis of Similarity test (ANOSIM; Clarke, 1993) was performed to access the significant difference in microbial community and groups (HP, MP, LP and DC). Similarity percentage analysis (SIMPER; Clarke & Warwick, 1994) was used to evaluate the contribution of each OTU on the differences seen in ANOSIM. ANOVA was used to tests whether diversity indices differ by experimental groups. To investigate if microbial community data and VFA concentrations change in the same way, a MANTEL test (Mantel, 1967) was performed. MANTEL was also used to evaluate if bacterial, archaeal and anaerobic fungal communities vary in an identical way. All these analyses were performed with the aid of Vegan package. For these analysis, $P<0.05$ were considered as significant.

Spearman correlation was used to correlate continuous variables (CH_4 emissions dDMI, dOMI, total VFA and concentrations of acetate, propionate and butyrate) and bacterial families and genera and archaeal and fungal genera relative abundances using the corrplot R package (Wei, 2012). For the bacterial community, only correlations significant at $P<0.05$ were added in the correlogram, except for the variable CH_4 in g/day, in which $P<0.1$ correlations were shown. For archaeal and anaerobic fungal communities any correlation found to be significant at $P<0.1$ were presented in the figure.

3. Results

3.1. Methane emissions and production traits

Methane emissions (Table 2) in g/day from MP were the greatest one ($P<0.05$). High producing cows and DC had similar emission and LP showed de lowest values ($P<0.05$). Considering the digestible fractions of DMI and OMI, DC had highest CH_4 emissions ($P<0.10$), followed by MP. High producing cows and LP did not differ in CH_4 emissions ($P>0.10$).

3.2. Sequencing and coverage metrics

After all filtering analyses using MOTHUR (v.1.35.0), the number of sequences reached was 214,676 for bacteria (mean= 11,298), 64,774 for archaea (mean= 3,409), and 12,544 for anaerobic fungi (mean= 660). For all amplicons, Good's Coverage of each sample was around 0.98.

3.3. Ruminal communities' composition

The most abundant bacterial phyla within all samples were Firmicutes (49.18%), Bacteroidetes (31.55%), and TM7 (8.36%) (Figure 1). Phyla SR1, Proteobacteria and Planctomycetes had abundances around 2%. The remaining 13 assigned phyla showed relative abundances lower than 1%. Relative abundances of Firmicutes largely varied among the groups and were greater for LP (56.30%) in comparison with HP (47.87%) and DC (42.15%) ($P<0.10$). The relative abundance of Firmicutes in MP (50.62%) did not differ from LP and HP but it was greater ($P<0.05$) than DC (42.15%). Bacteroidetes, TM7 and Firmicutes:Bacteroidetes did not differ between groups. Among Firmicutes sequences, genera *Ruminococcus*, *Butyrivibrio* and *Coprococcus* were the most abundant (8.25%, 6.97% and 3.46%). Sequences that did not reach the genus level corresponded to 49.21% and

unclassified sequences to 24.86%. Within Bacteroidetes sequences, genus *Prevotella* contributed the most, with 69.41% of relative abundance, followed by genus *CF231* in family Paraprevotellaceae with a relative abundance of 3.02%. Other genera relative abundances summed 1.89%, unclassified sequences were 3.01% and sequences that did not reach the genus level were 22.65%.

Methonobrevibacter was the most abundant genus in the archaeal community (93.43%), followed by *Methanosphaera* (5.32%) and *Vadin CA11* (1.23%). Dry cows had greater relative abundance of *Methanobrevibacter* (95.75% *versus* 90.04%) and lower abundance of *Methanosphaera* (3.01% versus 7.79%) than HP ($P<0.05$).

For the anaerobic fungal community, 73.19% of the sequences were unclassified, 21.95% were assigned as the genus *Caecomyces*, 4.16% to genus *Orpinomyces* and 0.70% to genus *Neocallimastix*. Anaerobic fungal relative abundances were similar between groups.

3.4. Communities richness and diversity

The number of species and the distribution of the taxa across all samples were evaluated using the indexes Chao1 for richness and Shannon for diversity. Comparing these indexes for the bacterial community, LP cows showed higher richness ($P<0.05$) than DC. Richness and diversity of archaeal and anaerobic fungal community were similar between groups.

3.5. Core microbiome

From the total OTU, 31.47% were observed in less than 10% of the samples, 24.02% were in 10 to 20% and 11.86% were present in 20 to 30% of the samples. This means that

67.35% of the OTU were shared by a small part of the samples. The bacterial taxa found to be ubiquitous across all samples in Holstein cows' rumen corresponded to 3.14% of the OTU or 32 OTU. Of the 32 OTU in the core, 21 belongs to phylum Firmicutes, eight to Bacteroidetes, two to phylum TM7 and one to Chloroflexi. Within Firmicutes, the OUTs in the core microbiome were from families Lachnospiraceae, Ruminococcaceae, Mogibacteriaceae and Clostridiaceae, all from order Clostridiales. Families Prevotellaceae, RF16 and Paraprevotellaceae represents phylum Bacteroidetes, order Bacteroidales. In TM7, family F16 and in Chloroflexi, family Anaerolinaceae. At genus level, the most important representatives of the core microbiome were *Prevotella*, *Ruminococcus*, *Butyrivibrio*, *Clostridium*, *Coprococcus*, *Shuttleworthia*, *CF231* and *SHD-231*.

Two OTU affiliated as genera *Methanobrevibacter* and *Methanosphaera* were present in all samples. None of the anaerobic fungi OTU are present in all samples. Almost 46% of the OTU are shared by less than 10% of the samples and a high proportion of the OTU (87%) are shared by only a few percentage of samples (up to 40%).

3.6. Influence of physiological and production traits in communities' composition and structure

Cows groups were observed to have different bacterial communities' composition ($P<0.05$) by ANOSIM. Several OTU were detected to influence bacterial community composition in each group and order Clostridiales was the mainly responsible for the observed changes (Table 3).

Archaeal community composition was not changed across groups. Anaerobic fungal community, on the other hand, was influenced by the groups ($P<0.05$). Two OTU affiliated

as family Neocallimastigaceae and species *Caecomyces communis* were the most important responsible for the differences found in community composition (Table 4). Neocallimatigaceae, represented by the OTU0001, was linked to as much as 33 to 35% of the changes in any comparison that involves DC. *Caecomyces communis* showed a higher impact in the difference among HP and MP cows.

Environmental variables were significant in clustering animals only in bacterial and anaerobic fungal communities (Figure 4 and Figure 5). Thus, archaeal nMDS figure was omitted. A clustering tendency can be observed in bacterial community, mainly in relation to DC and HP cows. Dry cows were clustered based on the CH_4 emissions and acetate:propionate ratio. For HP cows, dDMI and dOMI were the most important variables in clustering samples. In anaerobic fungal community, dDMI and dOMI were responsible for the HP and MP samples clustering together and acetate:propiponate ratio played this role for some DC and LP samples.

3.7. Correlation among bacterial, archaeal and anaerobic fungal families/genera and physiological and production traits

Spearman correlations were used to investigate the relationship between bacterial, archaeal and anaerobic fungal families and genera and CH_4 emissions and the other production parameters. The main results are shown in the correlogram (Figure 6).

Most bacterial families/genera that correlated with CH_4 emissions showed an inverse correlation. Families Coriobacteriaceae and Paraprevotellaceae and genus *Clostridium* correlated with CH_4 g/day ($r = -0.58$, $P<0.01$; $r = -0.46$, $P<0.05$; $r = -0.52$, $P<0.05$, respectively). Families Christensenellaceae, Coriobacteriaceae, Mogibacteriaceae,

Pirellulaceae, RFP12, RF16 and S24-7 and genera *Butyrivibrio*, *Clostridium*, *p-75-a5*, *Schwartzia* and *Treponema* showed inverse correlations with CH_4 in g/kg of DMI, g/kg of dDMI and g/kg of dOMI. The strongest ones were with families such as Coriobacteriaceae ($r = -0.87$ for g/kg of DMI, $r = -0.86$ for g/kg of dDMI and $r = -0.86$ for g/kg of dOMI, $P<0.01$) and genera *Butyrivibrio* ($r = -0.52$ for g/kg of DMI, $P<0.05$, $r = -0.65$ for g/kg of dDMI and $r = -0.64$ for g/kg of dOMI, $P<0.01$) and *Schwartzia* ($r = -0.58$ for g/kg of DMI, $r = -0.62$ for g/kg of dDMI and $r = -0.62$ for g/kg of dOMI, $P<0.01$). Two moderate positive correlations with CH_4 emissions were detected with families RF16 ($r = 0.60$ for CH_4 g/kg of dDMI, $P<0.01$ and $r = 0.56$ for g/kg of dOMI, $P<0.05$) and Succinivibrionaceae ($r = 0.56$ for CH_4 g/kg of dDMI, $P<0.05$ and $r = 0.58$ for g/kg of dOMI, $P<0.01$).

Families Christensenellaceae, Mogibacteriaceae and S24-7 and genera *Acetobacter*, *Butyrivibrio*, *Schwartzia* and *Treponema* were positively correlated with dDMI and dOMI. Strong negative correlations were found among these variables and family RF16 and genus *Coprococcus*.

Only few significant correlations between archaeal genera relative abundances and variables were detected. Surprisingly, *Methanobrevibacter* showed weak correlations with CH_4 emissions ($r = 0.35$, $P>0.10$), *Vadin Ca11* correlated with CH_4 in g/day ($r = 0.51$, $P<0.05$) and *Methanosphaera* negatively correlated with CH_4 in g/kg of DMI, g/kg of dDMI and g/kg of dOMI ($r = -0.43$, $P<0.1$; $r = -0.58$, $P<0.01$ and $r = -0.57$, $P<0.05$, respectively). *Methanobrevibacter* positively correlated with dDMI and dOMI and *Methanosphaera* showed negative correlations with these variables.

Among the anaerobic fungal OTU, genera *Caecomyces* was correlated with CH_4 g/day ($r = 0.49$, $P<0.05$) and with dDMI ($r = 0.40$, $P<0.1$). No significant correlation was found with CH_4 emissions.

Surprisingly, none of the bacterial, archaea and anaerobic fungi which correlate with CH_4 emissions showed significant correlations (P>0.1) with VFA and the individual concentrations of acetate, propionate and butyrate.

3.8. Covariation among bacterial, archaeal and anaerobic fungal communities and between these communities and volatile fatty acids

Covariation among bacterial, archaeal and anaerobic fungi communities was accessed via Mantel's test. Neither bacterial and archaeal, bacterial and anaerobic fungal or archaeal and anaerobic fungal communities covaried (P = 0.15, P = 0.14 and P = 0.20 respectively). Volatile fatty acids and microbial communities did not covary either (P = 0.18 for bacteria, P = 0.51 for archaea and P = 0.43 for anaerobic fungi).

4. Discussion

This research aimed to characterize the rumen microbiota of Holstein cows in lactation or not, in a functioning dairy system, located in a tropical country. In addition, to correlate the bacterial, archaeal and anaerobic fungal communities with CH_4 emissions and production traits, such as dDMI and dDMD was another objective.

With this research, we could show that cows in lactation or not in a tropical production system shared a core microbiome composed by *Prevotella*, *Ruminococcus*, *Butyrivibrio*, *Clostridium*, *Coprococcus*, *Shuttleworthia*, *CF231*, *SHD-231, Methanobrevibacter,* and *Methanosphaera*.

Methane emissions in relation to digestible fractions of the DMI and OMI were greater for DC group, followed by MP ($P<0.05$). High producing cows and LP cows showed lower CH_4 emissions than DC and MP, but they were similar to each other ($P<0.05$).

By the analysis of the nMDS plot we could conclude that the bacterial structure of DC and HP distinguish the most, basically in relation to CH_4 emissions, dDMI, dOMI and acetate:propionate ratio. In fact, DC showed higher CH_4 emissions and acetate:propionate ratio and lower propionate concentration than HP.

As noticed in previous studies evaluating the microbiota composition in rumen fluid samples (Tajima et al., 2007; Cunha et al., 2011; Lima et al., 2015), Firmicutes and Bacteroidetes were the most abundant phyla found within the rumen of Holstein cows. Firmicutes showed greater abundances in all groups and largely varied, being the relative abundance of this phylum similar between LP and MP and greater than HP and DC. These two last categories did not differ in Firmicutes relative abundance. Bacteroidetes did not show differences between HP, MP, LP and DC. *Ruminococcus* was the most abundant genus in Firmicutes, and *Prevotella* the most abundant genus in Bacteroidetes. Relative abundance of family Succinivibrionaceae in CH_4 emissions groups also was tested because this family was reported by Wallace et al. (2015) as more numerous in low-emitting beef cows. In disagreement with Wallace et al. (2015), the relative abundance of Succinivibrionaceae in DC was higher than in HP ($P<0.10$) and this family was positively correlated with CH_4 emissions.

Within archaeal community, the genera found to be the most abundant in Holstein cows in lactation or not was *Methanobrevibacter*, which is in agreement with other studies using ruminal fluid of cattle and sheep (Wright et al., 2008; King et al., 2011; Kumar et al., 2015). Findings from Wright et al (2008) are from Venezuela and did not differ from the

studies from temperate locations. Thus, it appears that the geographical location did not influence the archaeal abundance.

In line with other studies in ruminal fluid (Liggenstoffer et al., 2010; Kumar et al., 2015), unclassified sequences corresponded to more than half of the anaerobic fungi sequences (73.19 %). They might represent novel ruminal taxa of anaerobic fungi and they happen, probably, because of problems in assigning taxonomy rank to the sequenced reads (Kumar et al., 2015). Genus *Caecomyces* was the second most abundant in Holstein cows, as described previously by Liggenstoffer et al. (2010), which concluded that *Caecomyces* was the most abundant genus in data sets from domestic cattle. In somewhat of a contrast with prior studies, in the present study genera *Anaeromyces* (Liggenstoffer et al., 2010) and *Cyllamyces* (Ozkose et al., 2001; Kumar et al., 2015) were absent in the anaerobic fungal community of cows. Apparently, these genera are usually present in foregut ruminants but their populations establishment in rumen seems to be dependent on other factors, such as feed type and geographical location (Liggenstoffer et al., 2010). As these genera were not observed in the present study, this might be a difference in ruminal anaerobic fungal community due to the tropical environment in which the animals used were raised.

Because the widely described role of Firmicutes within the rumen is on fiber fermentation, we would expect that family and genera from this phylum would be associated with an increase in CH_4 emissions. However, families Christensenellaceae and Mogibacteriaceae and genera *Butyrivibrio*, *Clostridium*, *p-75-a5* and *Schwartzia* belong to Firmicutes and showed negative correlations with CH_4 emissions. This can be a result of the high relative abundance of Firmicutes in LP group, which showed unexpected low CH_4 emissions. Besides, in the case of family Mogibacteriaceae and genera *Butyrivibrio* and *Schwartzia,* the negative associations with CH_4 emissions might be due to their positive

correlation with dDMI and dOMI. Negative correlation among CH_4 emissions and *Clostridium* was reported before by Wallace et al. (2014), so we consider that unknown roles of uncultured bacteria in Firmicutes phylum could be responsible for these presumed unexpected findings. On the other hand, ruminal bacteria from phylum Bacteroidetes, such as Paraprevotellaceae, RF16 and S24-7 families, are supposed to be involved with an amilolytic fermentation pattern and, thus, with lower CH_4 emissions. In the present study, positive correlation among family RF16 and CH_4 emissions was an unexpected finding.

Families Christensenellaceae, Mogibacteriaceae and S24-7 and genera *Acetobacter*, *Butyrivibrio, Schwartzia* and *Treponema* correlated positively with dDMI and dOMI. Genus *Schwartzia* has been reported in rumen of cows in pasture (Van Gylswyk et al., 1997). It is characterized as asaccharolytic, anaerobic bacteria, which ferments succinate to propionate and do not ferment amino acids and peptides. In the first thought, it seems conversely that this genus could positively impact DMI due to the risk of acidification of the ruminal environment by its action. Nonetheless, when analyzing the complexity of the data obtained in this study, we can observe that the forage to concentrate ratio ranged from 90:10 to 50:50, leading to changes in the microbiota (Fernando et al., 2010). Thus, we noticed that cows fed higher concentrate amounts showed higher dDMI and dOMI and, therefore, we can assume that this correlation happened because of a crescent abundance of this bacteria with the change of the diet. On the other hand, family RF16 in phylum Bacteroidetes, known for its amylolytic fermentation, showed a strong negative correlation with dDMI and dOMI, evidencing that further research are required to better understand the complexity of rumen and its microorganisms.

Families or genera from Firmicutes were expected to increase DMI because of the ability of members of this phylum in degrading fibrous material during ruminal fermentation,

thus increasing flow of digesta and the stimulus to eat. The correlation with *Butyrivibrio* is a classic example of the expected association between Firmicutes and dDMI and dOMI and the negative correlation observed among *Coprococcus* and these variables was not expected. Out of the families/genera which were positively correlated with dDMI amd dOMI, Lima et al. (2015) reveal a great importance of the family Christensenellaceae (in Clostridiales) in rumen dynamics of Holstein cows, which is confirmed with the correlation observed herein. Family Mogibacteriaceae and genera *Schwartzia*, less discussed in literature, showed important correlations in the present study. In relation to rumen pH, genus *p-75-a5* (order Erysipelotrichales in Firmicutes) strongly correlated with this variable, but little is known about the action of *p-75-a5* in rumen.

No correlations were identified between *Methanobrevibacter* and CH_4 emissions. This result was completely unexpected, since it was shown before that *Methanobrevibacter* is associated with higher CH_4 emission (Wallace et al., 2015). Because of no correlations were detected, we believe that stronger correlations might be found in analysis of gene expressions rather than relative abundances. Another interesting finding of this study was the correlation between genus *Vadin CA11* and CH_4 g/day. Little is known about this genus. It is usually found in low abundances in rumen and there is no previous report in which it was positively associated with CH_4 emissions. *Methanosphaera* showed negative correlations with CH_4 emissions. Such finding corroborates with Kittelman et al. (2014). Thus, it can be observed that *Methanosphaera* were more efficient in producing CH_4 in low emitters cows than *Methanobrevibacter*. Further works focusing in finding out more about the role of these archaeal genus in the methanogenesis are required, especially *Vadin CA11* which has been reported only by a few authors (Wright et al., 2004, Kumar et al., 2015).

Genus *Methanobrevibacter* was negatively correlated with the digestible fractions of dDMI and dOMI, which can be confirmed by the higher relative abundance of this genus in high forage feed animals, such as DC. Genus *Methanosphaera* showed a moderate positive correlation with dDMI and dOMI. To our knowledge, such kind of correlation was not described before, unless by a tendency toward a positive relationship between total methanogens and DMD (Carberry et al. 2014). *Methanosphaera* is a methylotrophic methanogen, thus they use methylamines and methanol for CH_4 production and may have been influenced by the increasing presence of these substrates as a result of a greater digestibility.

Genus *Caecomyces* was correlated with CH_4 g/day which was expected, since anaerobic fungi are responsible for colonizing fibrous materials, making it more easily degradable, thus producing more acetate and, therefore, more CH_4. By the same token, positive correlations between this genus and dDMI and dOMI were predictable, because the greater degradability of the material increases the passage rate and, therefore, induce the consumption of more feed.

This study showed that bacterial, archaeal and anaerobic fungal communities did not covary, indicating that differences in HP, MP, LP and DC groups did not affect these communities in the same manner. Differences in the groups evaluated in this study did not make VFA and microbial communities vary in a similar way either, thus milk production, diet, CH_4 production and other differences between the cows' groups have little influence on the microbial communities. To our knowledge, similar results were not reported before.

In general, the most important results found in herein were the differences among DC and HP cows. Dry cows were found to emit more CH_4 when the digestible fractions of DMI and OMI were considered and, in comparison with HP, had greater relative abundance of

Methanobrevibacter and lower of *Methanosphaera*. Family Coriobacteriaceae was strongly and negatively correlated with CH_4 emissions and DC relative abundance of this family was lower than in HP. On the other hand, family Succinivibrionaceae showed a positive correlation with CH_4 emissions and greater relative abundances in DC. No differences in Firmicutes and Bacteroidetes relative abundances and, thus, in the Firmicutes:Bacteroidetes ratio were observed. Considering the fermentation patterns VFA concentrations were found to be greater for HP than DC, due especially to the greater proportion of propionate in HP cows' rumen. Acetate and butyrate concentrations were not different, although it was expected to detect greater acetate concentrations for DC, because of the diet they were fed and, consequently greater CH_4 produced by this category.

The results presented here demonstrate some important correlations with less abundant and uncommon reported in literature bacteria and archaea. However, such results need to be confirmed, so we would submit that further works should focus in a better understanding of the importance inside rumen of families such as Coriobacteriaceae, RF16, S24-7 and Christensenellaceae and genera such as *p-75-a5*, *Schwartzia*, *Treponema, Methanosphaera* and *Vadin CA11*. These findings can impact the future research in mitigating CH_4 emission through rumen microbiota manipulation. Supposing that these results will be confirmed, to find a way to favor these bacteria without losses in productive traits can help in producing meat and milk with a smaller carbon footprint.

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6. References

Association of Official Analytical Chemists (AOAC) 1990 Official Methods of Analyses of the AOAC, pp. 1–1230, 15 edition. Washington: Assoc OFF Agric Chemp.

Bauman, D.E., McCutcheon, S.N., Steinhour, W.D., Eppard, P.J., Sechen, S.J. (1985). Sources of variation and prospects for improvement of productive efficiency in the dairy cow: A review. J. Anim. Sci. 60:583–592.

Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18:117–143.

Clarke K.R., Warwick, R.M. (1994). Change in marine communities: an approach to statistical analysis and interpretation. Natural Environment Research Council, UK.

Connor, E.E., Hutchison, J.L., Olson, K.M., Norman, H.D. (2012). Opportunities for improving milk production efficiency in dairy cattle. J. Anim. Sci. 2012.90:1687–1694.

Cunha, I.S., Barreto, C.C., Costa, O.Y.A., Bomfim, M.A., Castro, A.P, Kruger, R.H., Quirino, B.F. (2011). Bacteria and Archaea community structure in the rumen microbiome of goats (Capra hircus) from the semiarid region of Brazil. Anaerobe, 17:118-124.

Carberry, C.A., Kenny, D.A., Kelly, A.K., Water, S.M. (2014). Quantitative analysis of ruminal methanogenic microbial populations in beef cattle divergent in phenotypic residual feed intake (RFI) offered contrasting diets. J Anim. Sci. and Biotechnol., 5:41.

Detmann, E., Souza, M.A., Valadares Filho, S.C., Queiroz, A.C., Berchielli, T.T., Saliba, E.O.S., Cabral, L.S., Pina, D.S., Ladeira, M.M., Azevêdo, J.A.G. (2012). Métodos para análise de alimentos, first ed. Suprema, Visconde do Rio Branco, MG, Brazil.

Fernando S.C., Purvis H.T., Najar F.Z., Sukharnikov L.O., Krehbiel C.R., Nagaraja T.G., Roe B.A. and DeSilva U. (2010). Rumen microbial population dynamics during adaptation to a high-grain diet. Appl Environ Microbiol 76, 7482–7490.

Henderson, G., Cox, F., Kittelmann, S., Miri, V.H., Zethof, M., Noel, S.J., Waghorn, G.C., Janssen, P.H. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. Plos One 597 8:e74787.

Intergovernmental Panel on Climate Change. Climate change 2014. Synthesis report 2014.

Johnson, K.A., Huyler, M., Westberg, H.H., Lamb, B. and Zimmerman, P. (1994). Measurement of methane emissions from ruminant livestock using a sulphur hexafluoride tracer technique. Environ. Sci. Technol. 28:359–362.

Johnson, K.A., Westberg, H. H., Michal, J. J. and Cossalman, M.W. (2007). The SF_6 tracer technique: Methane measurement from ruminants. In: Makkar H. S. and Vercoe P. (eds.) Measuring methane production from ruminants, pp. 33-67: Springer Netherlands.

King, E.E., Smith, R.P., St-Pierre, B., Wright, A.D.G. (2011). Differences in the rumen methanogen populations of lactating Jersey and Holstein dairy cows under the same diet regimen. Appl Environ Microbiol.77: 5682–5687.

Kittelmann, S., Seedorf, H., Walters, W.A., Clemente, J.C., Knight, R., Gordon, J.I., Janssen, P. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. PLoS ONE 8(2): p. e47879.

Kittelmann, S., Pinares-Patiño, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., McEwan, J.C., Janssen, P. (2014) Two Different Bacterial Community Types Are Linked with the Low-Methane Emission Trait in Sheep. PLoS ONE 9(7): e103171.

Kumar, S., Indugu N., Vecchiarelli, B., Pitta, D.W. (2015). Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to changes in diet and age in the rumen of dairy cows. Front. Microbiol. 6:781.

Liggenstoffer, A. S., Youssef, N. H., Couger, M., Elshahed, M. S. (2010). Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. ISME J. 4: 1225–1235.

Lima, F.S., Oikonomou, G., Lima, S.F., Bicalho, M.L.S., Ganda, E.K., Oliveira Filho, J.C., Lorenzo, G., Trojacanec, P., Bicalho, R.C. (2015). Prepartum and postpartum rumen fluid microbiomes: characterization and correlation with production traits in dairy cows. Appl Environ Microbiol 81:1327–1337.

Lodge-Ivey, S.L., Browne-Silva, J., Horvath, M.B. (2009). Technical note: Bacterial diversity and fermentation end products in rumen fluid samples collected via oral lavage or rumen cannula. J. Anim. Sci. 87:2333–2337.

Mantel N. (1967). The detection of disease clustering and a generalized regression approach. Cancer Res. 27:209–220.

Mendiburu, F. (2016). Agricolae: Statistical Procedures for Agricultural Research. R package version 1.2-4. http://CRAN.R-project.org/package=agricolae.

Menezes, A.B., Lewis, E., O'Donovan, M., O'Neill, B.F., Clipson, N., Doyle, E.M. (2011). Microbiome analysis of dairy cows fed pasture or total mixed ration diets. FEMS Microbiol. Ecol.78, 256–265.

Ozkose, E., Thomas, B.J., Davies, D.R., Griffith, G.W., Theodorou, M.K. (2001). Cyllamyces aberensis gen. nov. sp. nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. Can J Bot. 79: 666–673.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H. (2015). Vegan: community ecology package. URL http://vegan.r-forge.r-project.org/.

Primavesi, O., Frighetto, R.T.S., Pedreira, M.S., Lima, M.A., Berchielli, T.T., Demarchi, J.J.A.A., Manella, M.Q., Barbosa, P.F., Johnson, K.A. and Westberg, H.H. (2004). Técnica do gás traçador sf6 para medição de campo do metano ruminal em bovinos: Adaptações para o Brasil, p. 76. São Carlos - SP: Embrapa.

R Core Team (2015) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Reis, W.L.S., Detmann, E., Batista, E.D., Rufino, L.M.A., Gomes, D.I., Bento, C.B.P., Mantovani, H.C., Valadares Filho, S.C. (2016). Effects of ruminal and post-ruminal protein supplementation in cattle fed tropical forages on insoluble fiber degradation, activity of fibrolytic enzymes, and the ruminal microbial community profile. Animal Feed Science and Technology. 218, 1-16.

Siegfried, B. R.; Ruckemann, H.; Stumpf, G. (1984). Method for the determination of organic acids in silage by high performance liquid chromatography. Landwirtsch. Forsch, v.37, p.298.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F. (2009). Introducing Mothur: open-source, platform-independent, community supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.

Stevenson, D., and Weimer, P. (2007) 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: 165–174.

Stewart, C.S., Flynt, H.J., Bryant, M.P. (1997). The rumen bacteria, p. 10–72. In Hobson PN, Stewart CS (ed), The rumen microbial ecosystem. Blackie Academic and Professional, New York, NY.

Tajima, K., Nonaka, I., Higuchi, K., Takusari, N., Kurihara, M., Takenaka, A., Mitsumori, M., Kajikawa, H., Aminov, R.I. (2007). Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. Anaerobe 13:57-64.

Van Gylswyk, N.O., Hippe, H., Rainey, F.A. (1997). Schwartzia succinivorans gen. nov., sp. nov., another ruminal bacterium utilizing succinate as the sole energy source. Int. J. Syst. Bacteriol. 47:155–159.

Wallace, R.J., Rooke, J.A., Duthie, C., Hyslop, J.J., Ross, D.W., McKain, N., Souza, S.M., Snelling, T.J., Waterhouse, A., Roehe, R. (2014). Archaeal abundance in post-

mortem ruminal digesta may help predict methane emissions from beef cattle. Scientific Reports, 4:5892.

Wallace, R.J., Rooke, J.A., McKain, N., Duthie, C.A., Hyslop, J.J., Ross, D.W., Waterhouse, A., Watson, M., Roehe, R. (2015). The rumen microbial metagenome associated with high methane production in cattle. BMC Genomics. 16:839.

Wei, T. (2012) Package 'Corrplot' – Visualization of a correlation matrix v0.60. cran.rproject.org. URL http://cran.r-project.org/web/packages/corrplot/corrplot.pdf.

Wright, A.D.G., Williams, A.J., Winder, B., Christophersen, C.T., Rodgers, S.L., Smith, K.D. (2004). Molecular diversity of rumen methanogens from sheep in Western Australia. Applied and Environmental Microbiology. 70:3, 1263-1270.

Wright, A.D.G., Ma, X., Obispo, N.E. (2008). Methanobrevibacter phylotypes are the dominant methanogens in sheep from Venezuela. Microb. Ecol. 56:390–394.

Yan, T., Mayne, C.S., Gordon, F.G., Porter, M.G., Agnew, R.E., Patterson, D.C., Ferris, C.P., Kilpatrick, D.J. (2010). Mitigation of enteric methane emissions through improving efficiency of energy utilization and productivity in lactating dairy cows. J. Dairy Sci. 93:2630–2638.

Table 1. Percentage of ingredients and chemical composition of concentrate and experimental diet

Item	Concentrate1	Diet		Concentrate2	Diet	
		HP	MP		LP	DC
Corn silage	-	41.50	70.00	-	80.00	90.00
Cost-cross hay	-	8.50	-	-	-	-
Corn meal	40.80	20.40	12.24	26.00	5.20	2.60
Soybean meal	53.30	26.65	15.99	34.40	6.88	3.44
Wheat bran	-	-	-	31.80	6.36	3.18
Urea	-	-	-	2.92	0.58	0.29
Ammonium sulfate	-	-	-	0.32	0.06	0.03
Mineral mix	3.74	1.87	1.12	4.50	0.90	0.45
Sodium bicarbonate	1.42	0.71	0.43	-	-	-
Magnesium oxide	0.71	0.34	0.21	-	-	-
Vitamin mix	0.10	0.05	0.03	0.10	0.02	0.01
Chemical composition						
Dry matter	88.00	62.25	50.22	89.00	45.02	39.53
Crude protein	31.00	19.63	14.84	31.00	12.09	9.72
Ether extract	2.00	2.12	2.13	3.00	2.21	2.11
Neutral detergent fiber	31.00	41.85	45.70	27.00	55.93	59.54
Mineral mix	8.00	6.44	5.90	9.00	6.62	6.32

Table 2. Methane emissions and production traits of Holstein cows in lactation or not

Sample	CH_4 g/day	CH_4 g/kg DMI	CH_4 g/kg dDMI	CH_4 g/kg dOMI	DMI (kg)	DMD	dDMI (kg)	dOMI (kg)	MP (L/day)	Total VFA (mmol/L)	Acetate (mmol/L)	Propionate (mmol/L)	Butyrate (mmol/L)	A:P
HP1	397.03	25.3	28.32	30.77	15.69	0.77	14.02	12.90	31.77	113.73	68.24	25.52	11.23	2.67
HP2	274.37	15.05	16.65	18.21	18.23	0.78	16.47	15.07	32.5	122.44	71.34	32.45	11.46	2.20
HP3	284.52	20.26	23.00	24.96	14.04	0.77	12.37	11.40	19.74	141.52	78.80	39.51	14.12	1.99
HP4	214.81	19.55	22.78	24.69	10.99	0.75	9.43	8.70	17.96	128.74	75.54	33.76	11.92	2.24
HP5	260.38	15.5	17.77	19.35	16.80	0.74	14.65	13.46	27.34	115.92	68.43	28.25	12.01	2.42
Mean	286.22B	19.13B	21.70C	23.60C	15.15B	0.76A	13.39A	12.31A	25.86A	124.47A	72.47A	31.90A	12.15	2.31B
MP1	385.15	25.61	33.57	36.30	15.04	0.68	11.47	10.61	14.4	108.27	68.39	22.22	10.76	3.08
MP2	395.67	21.41	26.55	28.40	18.48	0.73	14.90	13.93	18.52	94.45	60.15	20.83	8.19	2.89
MP3	393.52	25.39	31.41	33.78	15.50	0.73	12.53	11.65	14.24	98.98	61.86	20.44	9.91	3.03
MP4	297.22	21.73	27.55	29.71	13.68	0.71	10.79	10.00	16.4	122.35	76.81	24.55	13.07	3.13
MP5	378.05	21.27	27.17	29.03	17.77	0.7	13.91	13.02	14.14	117.30	74.74	23.05	11.86	3.24
Mean	369.92A	23.08AB	29.25B	31.44B	16.09A	0.71B	12.72A	11.84A	15.54B	108.27AB	68.39A	22.22B	10.76	3.07A
LP1	174.81	15.95	24.49	25.52	10.96	0.65	7.14	6.85	10.8	89.15	60.70	16.31	7.30	3.72
LP2	129.96	10.79	16.67	17.96	12.05	0.65	7.80	7.23	9.06	66.35	51.25	21.39	16.63	2.40
LP3	201.42	13.13	19.21	20.17	15.34	0.68	10.49	9.99	12.56	97.27	64.40	17.48	8.76	3.68
LP4	192.54	13.27	17.72	18.92	14.51	0.75	10.87	10.18	9.8	49.28	54.74	20.95	16.48	2.61
Mean	174.68C	13.29C	19.52C	20.64C	13.22ABC	0.68C	9.07B	8.56B	10.56C	75.51C	57.77B	19.04B	12.29	3.10A
DC1	262.64	21.6	34.58	36.23	12.16	0.62	7.60	7.25	NA	106.17	68.71	22.52	9.43	3.05
DC2	249.05	21.09	30.74	32.05	11.81	0.69	8.10	7.77	NA	100.79	65.99	20.63	9.02	3.20
DC3	296.21	30.16	46.00	48.47	9.82	0.66	6.44	6.11	NA	103.65	65.88	22.00	8.95	2.99
DC4	301.87	27.05	40.24	42.46	11.16	0.67	7.50	7.11	NA	98.68	65.39	18.85	8.89	3.47
DC5	276.96	25.53	38.97	41.24	10.85	0.65	7.11	6.72	NA	97.37	63.40	20.91	8.63	3.03
Mean	277.35B	25.09A	38.10A	40.09A	11.16C	0.66C	7.35B	6.99B	-	101.33B	65.87AB	20.98B	8.98	3.15A

CH_4= methane; DMI= dry matter intake; DMD= dry matter digestibility; dDMI= digestible dry matter intake; dOMI= digestible organic matter intake; MP= milk production; VFA= volatile fatty acids; NA= not available. HP = high-producing; MP = medium-producing; LP = low-producing and DC= dry cows. Means followed by different letters in a column are different by Tukey test ($P<0.10$).

Table 3. Percentages of the importance of the OTU in changes in bacterial community composition of Holstein cows

OTU #	Order	Lowest classification	HP x MP	HP x LP	HP x DC	MP x LP	MP x DC	LP x DC
Otu01	CW040	F16 (F)	0.06	0.07	0.06	0.07	0.07	0.07
Otu02	Bacteroidales	*Prevotella* (G)	0.05	0.03	0.03	0.09	0.05	0.06
Otu03	Clostridiales	Lachnospiraceae (F)	-	-	0.03	-	0.05	0.03
Otu04	Clostridiales	Clostridiales (O)	0.05	0.05	0.06	-	-	-
Otu05	Clostridiales	Ruminococcus (F)	-	-	-	-	0.03	0.03
Otu06	Clostridiales	Clostridiales (O)	-	0.03	-	0.03	0.03	0.03
Otu07	CW040	F16 (F)	-	-	-	-	-	-
Otu08	Clostridiales	Ruminococcaceae (F)	0.04	0.03	-	-	0.03	0.03
Otu10	-	SR1 (P)	-	-	0.03	-	0.03	0.03

HP = high-producing; MP = medium-producing; LP = low-producing and DC= dry cows.

Table 4. Percentages of the importance of the OTU in changes in anaerobic fungal community composition of Holstein cows

OTU #	Order	Lowest classification	HP x MP	HP x LP	HP x DC	MP x LP	MP x DC	LP x DC
Otu01	Neocallimastigales	Neocallimastigaceae (F)	0.21	0.26	0.33	0.28	0.35	0.33
Otu02	Neocallimastigales	*Caecomyces_communis* (S)	0.24	0.16	0.16	0.18	0.17	0.15
Otu03	Neocallimastigales	Neocallimastigaceae (F)	0.21	0.17	0.17	0.23	0.23	-
Otu05	unclassified	unclassified		0.11	-	0.12	-	0.14
Otu06	unclassified	unclassified	0.12	-	0.08	-	-	-
Otu11	Neocallimastigales	*Orpinomyces* (G)						0.09

HP = high-producing; MP = medium-producing; LP = low-producing and DC= dry cows.

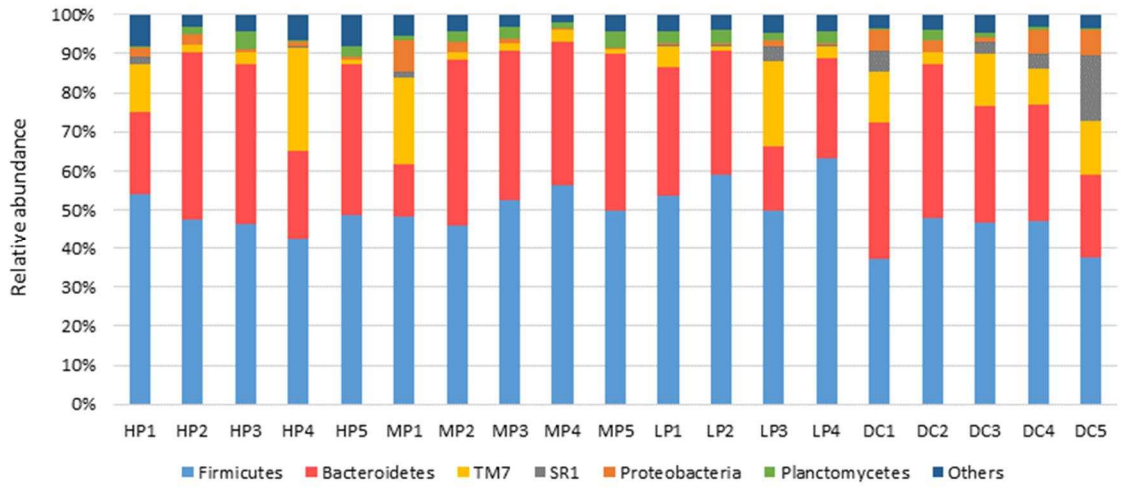

Figure 1. Relative abundance of bacterial community of Holstein cows at phylum level. HP = high-producing; MP = medium-producing; LP = low-producing and DC= dry cows.

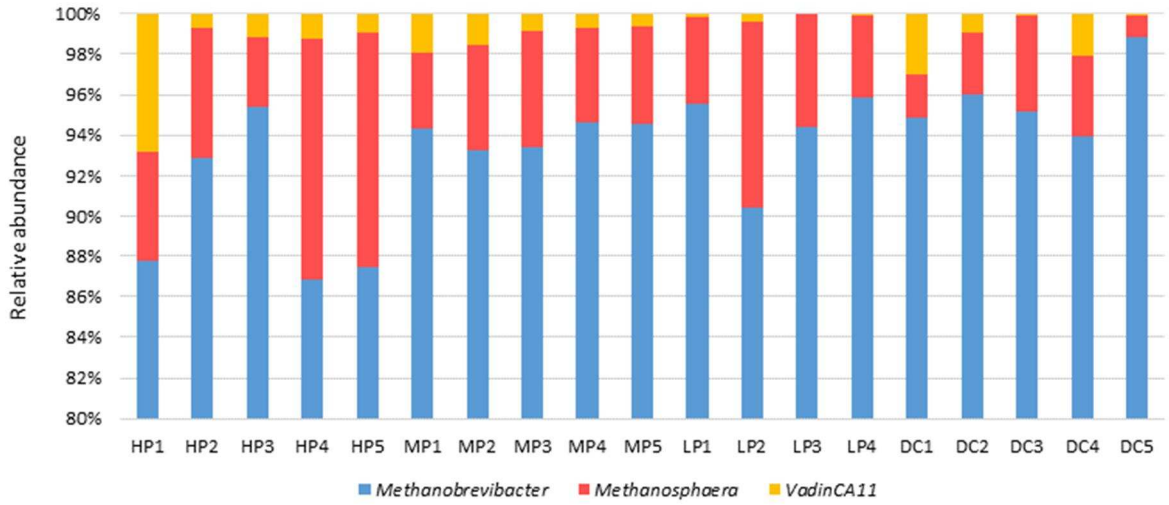

Figure 2. Relative abundance of archaeal community of Holstein cows at genera level. HP = high-producing; MP = medium-producing; LP = low-producing and DC= dry cows.

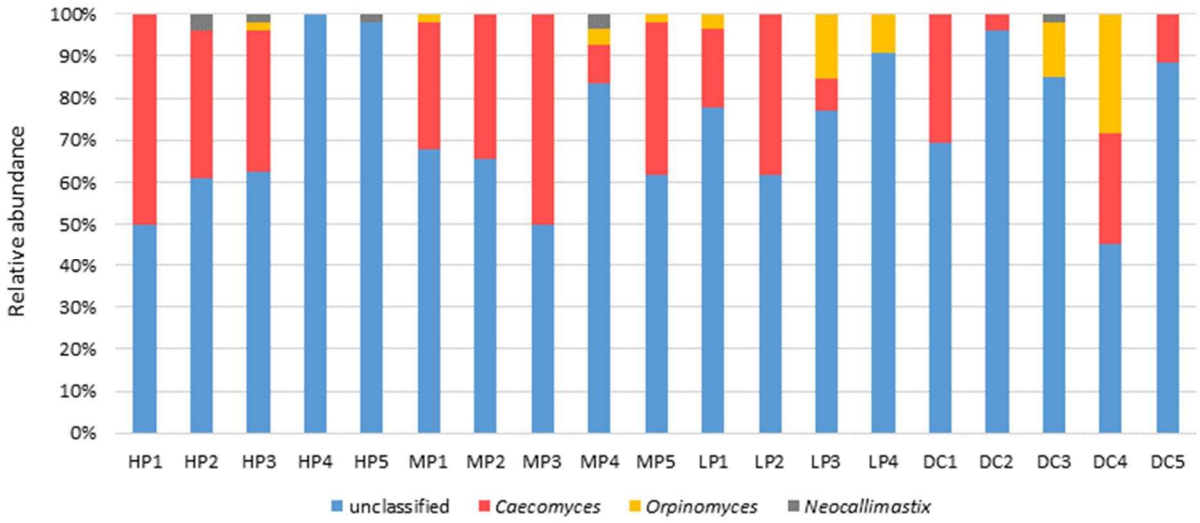

Figure 3. Relative abundance of anaerobic fungal community of Holstein cows at genus level. HP = high-producing; MP = medium-producing; LP = low-producing and DC= dry cows.

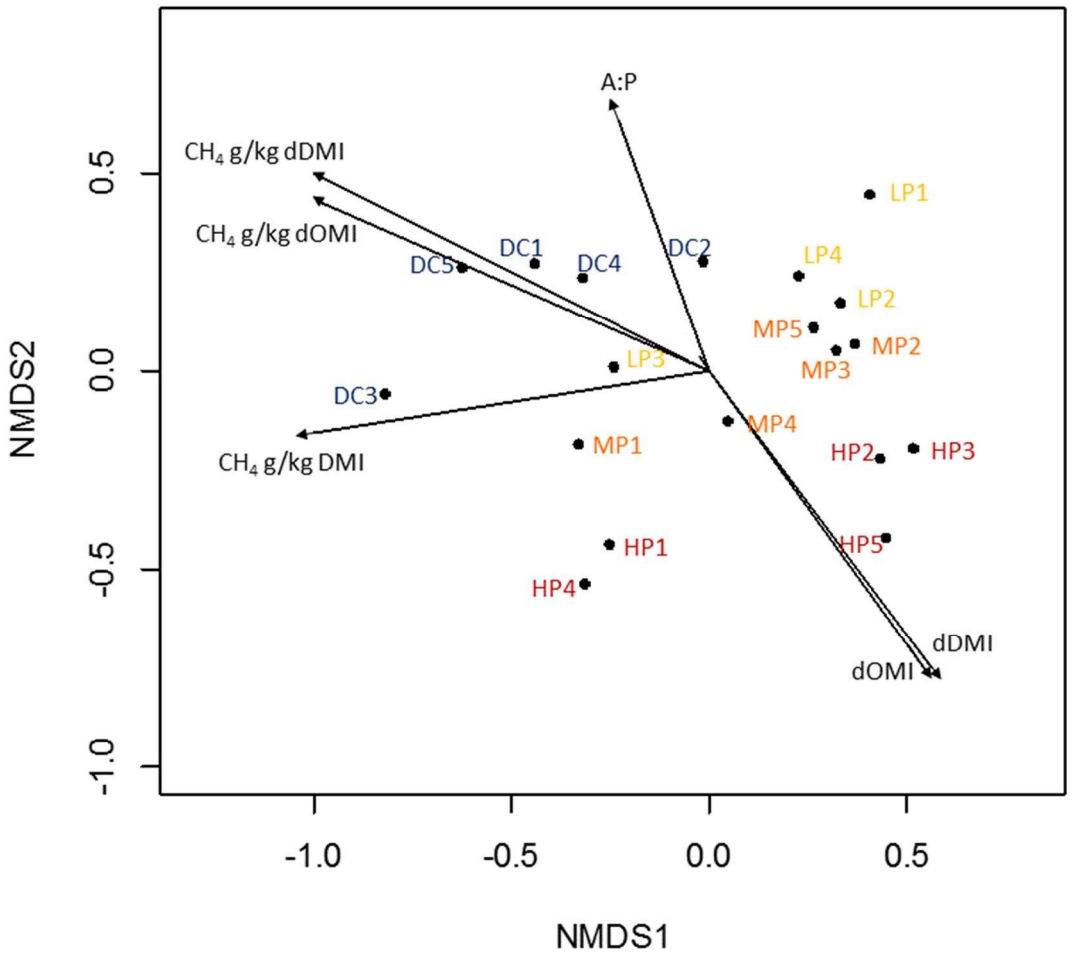

Figure 4. Non-metric multidimensional scaling plot of Bray-Curtis dissimilarity index of bacterial community structure. HP = high-producing; MP = medium-producing; LP = low-producing and DC= dry cows. Each data point represents the microbial community of each evaluated cow.

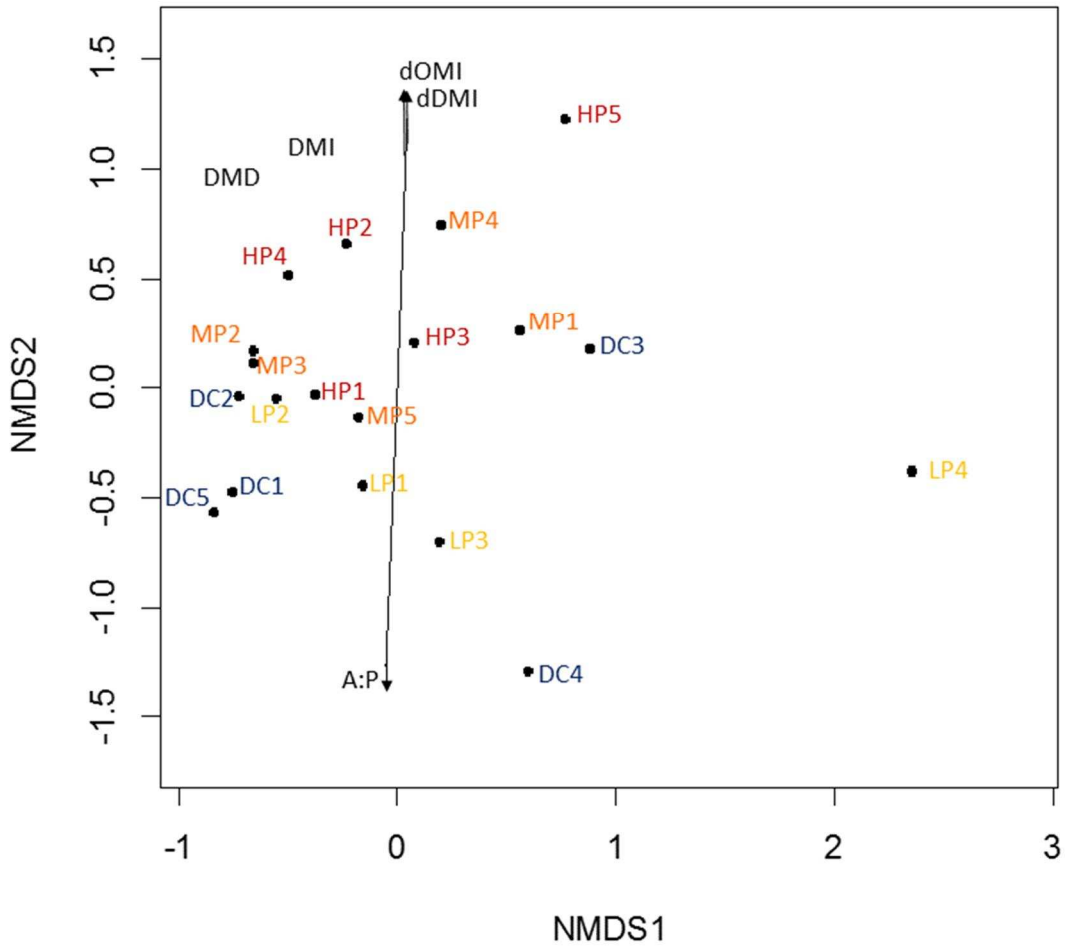

Figure 5. Non-metric multidimensional scaling plot of Bray-Curtis dissimilarity index of anaerobic fungal community structure. HP = high-producing; MP = medium-producing; LP = low-producing and DC= dry cows. Each data point represents the microbial community of each evaluated cow.

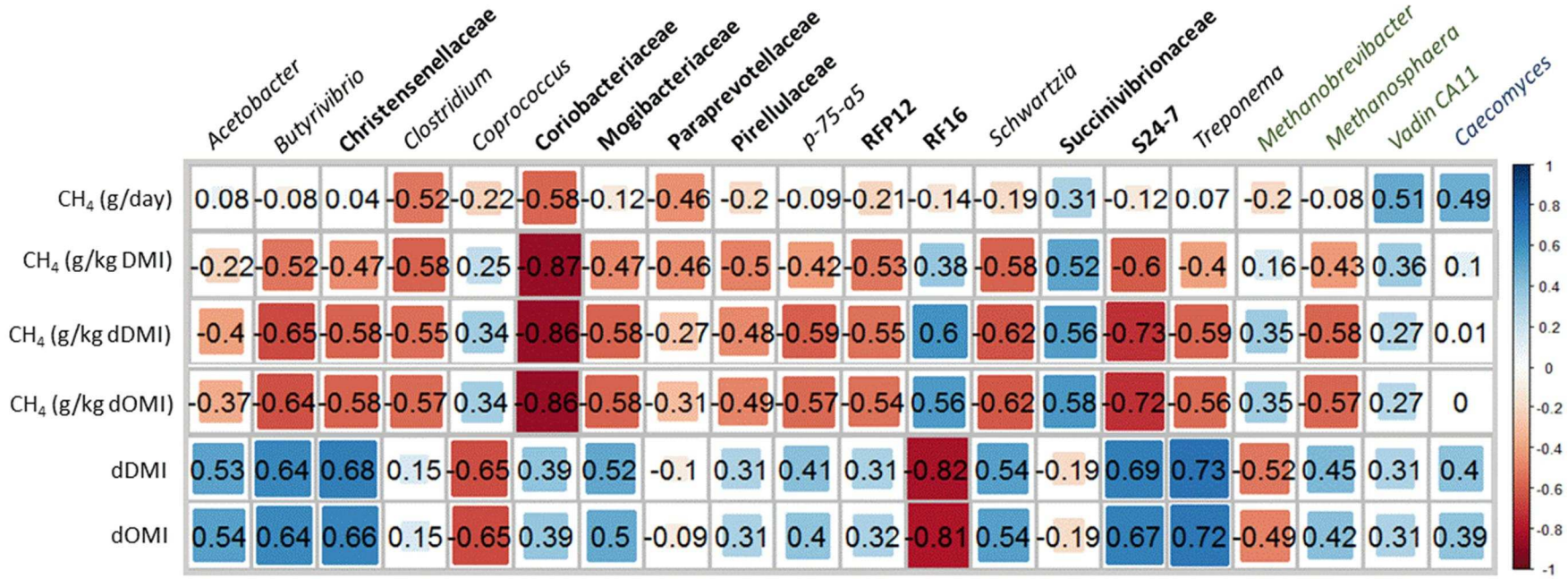

Figure 6. Correlation between CH_4 emissions and other production parameters and relative abundance at family and genus taxonomic level. Spearman linear correlation matrix of selected family/genus across all samples. Names in bold means family, the other refers to genus taxonomic level. Names in black, green and blue means, respectively, bacteria, archaea and anaerobic fungi. CH_4 = methane emissions; dDMI = digestible dry matter intake; dOMI = digestible organic matter intake.

CHAPTER 3

GREENHOUSE GASES INVENTORY AND CARBON BALANCE OF TWO DAIRY SYSTEMS OBTAINED FROM TWO METHANE-ESTIMATION METHODS

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Abstract

The adoption of carbon inventories for dairy farms in tropical countries based on models developed from animals and diets of temperate climates is questionable. Thus, the objectives of this study were to estimate enteric methane (CH_4) emissions through the SF_6 tracer gas technique and through equations proposed by the Intergovernmental Panel on Climate Change (IPCC) Tier 2 and to calculate the inventory of greenhouse gas (GHG) emissions from two dairy systems. In addition, the carbon balance of these properties was estimated using enteric CH_4 emissions obtained using both methodologies. In trial 1, the CH_4 emissions were estimated from seven Holstein dairy cattle categories based on the SF_6 tracer gas technique and on IPCC equations. The categories used in the study were prepubertal heifers (n = 6); pubertal heifers (n = 4); pregnant heifers (n = 5); high-producing (n = 6); medium-producing (n = 5); low-producing (n = 4) and dry cows (n = 5). Enteric methane emission was higher for the category comprising prepubertal heifers when estimated by the equations proposed by the IPCC Tier 2. However, higher CH_4 emissions were estimated by the SF_6 technique in the categories including medium- and high-producing cows and dry cows. Pubertal heifers, pregnant heifers, and low-producing cows had equal CH_4 emissions as estimated by both methods. In trial 2, two dairy farms were monitored for one year to identify all activities that contributed in any way to GHG emissions. The total emission from Farm 1 was 3.21 t CO_2e/animal/yr, of which 1.63 t corresponded to enteric CH_4. Farm 2 emitted 3.18 t CO_2e/animal/yr, with 1.70 t of enteric CH_4. IPCC estimations can underestimate CH_4 emissions from some categories while overestimate others. However, considering the whole property, these discrepancies are offset and we would submit that the equations suggested by the IPCC properly estimate the total CH_4 emission and carbon balance of the properties.

Thus, the IPCC equations should be utilized with caution, and the herd composition should be analysed at the property level. When the carbon stock in pasture and other crops was considered, the carbon balance suggested that both farms are sustainable for GHG, by both methods. On the other hand, carbon balance without carbon stock, by both methods, suggests that farms emit more carbon than the system is capable of stock.

Keywords: Methane emission, carbon equivalent, greenhouse gases, dairy cattle, milk production, ruminants

1. Introduction

Despite the great importance of milk and beef livestock for national and global economic scenarios, these activities are responsible for many environmental issues such as emission of greenhouse gases (GHG) (Berndt & Tomkins, 2013). Concerns about global warming have increased the pressure on the dairy and beef cattle systems in Brazil and worldwide to promote an environmentally sustainable livestock production. Thus, research with a focus on determining GHG emissions from the livestock sector is essential in order to develop more sustainable practices.

According to the Brazilian national inventory, the methane (CH_4) emissions from enteric fermentation and animal waste from cattle amount to 57 million tons of carbon (Lima et al., 2002). However, the application of inventories in dairy production systems in tropical countries based on methods developed from animals raised in temperate climates is doubtful, since the products and by-products of digestive processes and their interaction with the environment differ considerably across tropical countries (Kurihara et al., 1999), particularly when considering the different forages consumed by the animals.

Developing GHG inventories is important for determining the emission profile of the livestock sector; for the establishment of reduction targets; and to stimulate the adoption of sustainable alternatives in tropical climates (Santos et al., 2010). Despite their importance, the use of inventories on farms remains incipient and insufficient to provide more information of GHG emissions and carbon footprint estimation, which can be used as an environmental management tool when associated with parameters such as productivity, feed characteristics, feed intake and manure management system. In addition, inventory calculations by 2013 were not typically associated with estimates of the carbon balance of the property, and published data is contradictory as to whether farms in pasture-based systems are big carbon emitters or carbon sequesters. For example, some research has shown that these systems are capable of sequestering carbon from the atmosphere and storing it in soils (Zen et al., 2008).

Given the above considerations, the aim of this study was to evaluate the CH_4 emissions of different categories of dairy cattle by the SF_6 tracer gas technique and as recommended by the IPCC (2006). Additionally, the objective was to calculate the inventory of GHG emissions from two dairy systems, and to estimate the carbon balance of these properties considering the enteric CH_4 emissions estimated by the IPCC (2006) equations compared to the SF_6 tracer gas technique.

2. Material and methods

The present study was set in two trials. In the first trial the SF_6 tracer gas technique was used to estimate CH_4 emissions from different categories of dairy cattle. Using productivity and feed intake data from these animals, CH_4 emissions were estimated as suggested by the IPCC (2006) Tier 2 equations, and both estimations were compared. In the second trial, a massive data collection was made in two dairy farms to estimate the total GHG

emitted by these farms and to estimate carbon balance. Animals' production level was similar to Trial 1. Since it was not possible to measure enteric CH_4 emissions from animals in farms, data regarding enteric CH_4 emissions used in these calculations were those obtained by both methodologies used in Trial 1. In this way, it was possible to compare the carbon balance estimation using the SF_6 technique and the IPCC (2006) equations in estimating CH_4 emissions derived from enteric fermentation.

2.1. Trial 1

The trial was conducted in the facilities of the Unit for Teaching, Research, and Extension on Dairy Cattle (UEPE-GL) of the Federal University of Viçosa (UFV), in Viçosa-MG, Brazil. The project was approved by the Ethics Committee in the Use of Production Animals from the UFV (protocol number 99/2014).

Holstein cows in different stages of lactation and Holstein heifers of different ages were divided into the following groups: prepubertal heifers, 200 ± 13.66 kg (n = 6); pubertal heifers, 350 ± 10.29 kg (n = 4); pregnant heifers, 450 ± 23.59 kg (90 d post artificial insemination, n = 5); high-producing (27.09 ± 2.74 L milk/d; n = 6); medium-producing (15.54 ± 0.85 L milk/d; n = 5); low-producing (10.56 ± 0.76 L milk/d; n = 4) and dry cows (n = 5). All animals were housed in individual tie stalls.

The trials were carried out separately for each production phase. The first five days of each trial were used for the adaptation of the animals. Gas collections from animals' eructation for evaluation of CH_4 emissions began on the sixth day. The collection period of each trial lasted five days for collection of gases and evaluation of consumption. Milk production was recorded and all animals were weighed, without previous food deprivation,

at the beginning and end of each trial.

Diet was supplied twice daily, at 07 a.m. and 03 p.m., and adjusted to keep the refusals at around 5 to 10% of the total supplied. All concentrates were based on corn, soybean meal and minerals and the forage:concentrate ratios (Table 1) were similar to those of the farms evaluated in Trial 2 of this study. Water was available *ad libitum* to the animals.

After the sixth day of each trial, the forage and the refusals from each animal were sampled, and the daily amounts of feed supplied and refusals were recorded. All samples were processed and evaluated for the dry matter (DM) content according to AOAC (1990), and the results were used for the calculation of the dry matter intake (DMI).

The CH_4 emissions were measured using the sulphur hexafluoride (SF_6) tracer gas technique (Johnson et al., 1994) with adaptations proposed by Primavesi et al. (2004). Briefly, the flow of CH_4 released by the animal was calculated in relation to the flow of SF_6, measured from the SF_6 release rate from the permeation capsule lodged in the rumen and from the concentrations of CH_4 and SF_6 in gas samples (Johnson et al., 2007). On the 4th day of each trial, the animals were forced to ingest one permeation capsule with known SF_6 release rate. To collect the gases, a PVC-tube collection-container yoke was used with a halter coupled to a collecting apparatus. Before the beginning of each collection day, the yokes went through a cleaning process, were evacuated at approximately –14 PSI, and then placed on the neck of each animal and coupled to the halter using a quick attachment. After 24 h, the yokes that were on the animals were switched for clean and evacuated yokes and the pressure of each yoke was recorded. The yokes containing samples of gases were pressurized with approximately 14 PSI of N_2 gas 99.999% purity. After a minimum period of 1 h for homogenization of the gases inside the yoke, the pressure was recorded once again, and the samples were collected in previously evacuated Exetainer® tubes.

Analyses of CH_4 and SF_6 levels were performed at the Laboratory of Gas Chromatography of EMBRAPA Dairy Cattle, in Juiz de Fora-MG. The results were expressed in g CH_4/animal/day, g CH_4/kg DM consumed, and g CH_4/kg product (milk or weight gain). The results were compared to data estimated by the IPCC (2006) by Student's t-tests at a 5% significance level.

2.2 Trial 2

An inventory of GHG emissions was taken on two farms from Minas Gerais State with different characteristics and were prepared based on the guide proposed by the International Dairy Federation (IDF, 2010). All activities in dairy production that were related in any way to GHG emissions were recorded for an entire year (April 2012 to March 2013). In order to obtain a meaningful data set, an exhaustive data collection was made through visits that occurred every 14 days. The enormous work involved in the data collection precluded the addition of more farms to this study. The following factors were considered: emissions from enteric CH_4, management of animal waste, electrical energy, fossil fuels, nitrogen (N) fertilization of pastures, eucalyptus (silvopastoral system), and agricultural crops that were used to feed the cattle (corn, sugarcane, and grass).

Farm 1 is located in the municipality of Coimbra (20º51'24"S and 42º48'10"W). The climate of the region consists of two well-defined seasons: summer (hot and rainy) and winter (cold and dry) with an annual average temperature of 21.6 ºC and an annual precipitation of 1,283 mm. The property has total area of 202 ha consisting of 53 ha for dairy farming of which 38.5 ha are used for pasture; the corn crop occupies an area of 9.5 ha; sugarcane 3.8 ha; and pasture for cut (*Pennisetum purpureum* cv. Napier) 1.2 ha. In addition, the property

has three artificial dams, 61 ha of coffee plantations, and 88 ha of legal reserve area.

The production system in Farm 1 is intensive, using semi-confined animals, where the cows were kept in seven groups indoors during the day and were pasture-grazed during the night. Lactating cows were fed pasture, corn silage, sugarcane (enriched with urea), and concentrate. Dry cows were fed pasture, sugarcane, and 1 kg of concentrate. Prepartum animals were fed corn silage and 1 kg of concentrate. All concentrates were based on corn, soybean meal and minerals. The annual average herd size was 113 animals, consisting of 58 cows (43 in lactation), 55 heifers, 6 female calves and 2 breeding bulls. The property utilizes Holstein animals and some crossbreeds with the Jersey breed (Table 2).

The fertilizer used was composed by nitrogen, phosphorus and potassium (NPK) in different contents expressed in parenthesis. Planting and fertilization during the evaluated period occurred as follows: for the corn crop, 700 kg/ha NPK (08-24-12) was applied at planting plus 700 kg/ha NPK (30-00-10), and 200 kg urea as top dressing. Sugarcane received 400 kg NPK (08-28-16) at planting and 150 kg NPK (18-06-24) as topdressing. For the grass (Elephant grass), 250 kg/ha NPK (08-28-16) was applied at planting plus 100 kg/ha NPK (18-06-24) as top dressing. For the pasture, 250 kg/ha NPK (08-28-16) was applied at planting plus 500 kg/ha NPK (20-00-20) and 400 kg urea as top dressing.

Farm 2 is located in the municipality of Porto Firme (20º38'54"S and 43º05'18"W, 640 m altitude). The climate of the region of Porto Firme is a Cwb type, according to the Köppen system (i.e., mesothermal with hot and rainy summers and cold and dry winters). The average annual temperature is 21.8 ºC, with an annual precipitation of 1,221 mm. Farm 2 has a total area of 15.6 ha of which 7.8 are utilized in dairy farming (1.03 ha of pasture, 2.07 ha of sugarcane and 4.7 ha of corn for silage). The production system is semi-intensive. The herd was fed on the basis of pasture supplemented with sugarcane enriched with urea

(1% fresh weight), corn silage, and concentrate (based on soybean meal, corn and minerals). The lactating cows were placed in 38 paddocks of 270 m^2 of Mombaça grass (*Panicummaximum* Jacq.) in a rotational system (silvopastoral system – Mombaça grass and eucalyptus). At the time of the study, the herd consisted of 10 cows in lactation and 2 dry cows (Table 2).

For the planting of the eucalyptus in the silvopastoral system, NPK (06-30-06) was applied at 0.150 kg/furrow. Three months after planting, the eucalyptus was top-dressed with the application of NPK (20-05-20) at 0.160 kg/plant. For the corn crop, 500 kg/ha NPK (08-24-12) was applied at planting, 500 kg/ha NPK (30-00-10), and 200 kg urea were applied as topdressing. For the pasture, the producer utilized 187.5 kg/ha NPK (08-28-16) at planting plus 250 kg/ha NPK (20-00-20) and 250 kg urea as top dressing. For the sugarcane, 180 kg NPK (08-28-16) were used at planting plus 150 kg NPK (20-05-20) as top dressing.

Calculations

All the calculations followed those described by the IPCC (2006), the same used in the Brazilian Inventory, using Tier 2 for all estimations. All the Equations used can be found in the Appendix section. The enteric CH_4 was estimated by Equations 1–10, and the emissions originating from the management of the animal waste were estimated using Equations 11–13. Thus, the total CH_4 emission was calculated as the sum of CH_4 emissions from enteric fermentation and from animal wastes.

The total emissions of nitrous oxide (N_2O) were calculated as the sum of the direct and indirect emissions plus emissions from the deposition of animal wastes in the pasture (Equation 14; IPCC, 2006). The direct emissions of N_2O are those produced mainly by the

addition, by the producer, of animal manure in pastures as fertilizer, use of synthetic fertilizers, growing of nitrogen-fixing crops, incorporation of crop residues into the soil, and mineralization of N due to the cultivation of organic soils. The N_2O emission originating from N fertilization (planting and topdressing fertilization of all crops associated with milk production) in agricultural soils was estimated using Equations 15–19 (IPCC, 2006). Fertilization at planting pasture was divided by 15, which is the average period (years) demanded for a new planting. The indirect emissions of N_2O refer to the portion of N incorporated into the soil that is volatized as NH_3 and NO_x and that is also lost by leaching (Equations 20–22). Emissions of N_2O_p by wastes were estimated according to Equation 23 (IPCC, 2006).

The Global Warming Potential (GWP) used in the conversion of CH_4 and N_2O emissions in carbon equivalent emissions (CO_2e) was 24 kg CO_2e/kg CH_4 and 298 kg CO_2e/kg N_2O.

The soil at both properties was prepared by ploughing once and disking twice. The fuel consumption was calculated according to the technical specificities of the agricultural machines, which indicate the use of 16.56 L/ha of diesel for ploughing and 21.71 L/ha of diesel for disking.

For preparation of silage, during the dry season, the average work rate of forage harvesters (model PN Plus 2000) was adopted, which was 1.0 ha of crop (corn, sugarcane, grass) per hour of operation, with an average consumption of 17 L/h. The carbon equivalent emission by mobile combustion, in t CO_2e, were calculated according to Equation 24.

The CO_2 emissions from the power consumption of the electrical grid are associated with the irrigation of crops and mechanized milking of lactating cows. Irrigation was used only on Farm 1. The calculation was made considering an irrigation yield of 40.8 kWh/ha/d

by the pump (Dancor CAM W10 3 CV) and a period of 100 days of sprinkler irrigation per year. In the milking operations, a 3-set Alfalaval milking machine was used on Farm 1 and a 2-set Delaval milking machine was employed on Farm 2, with an energy consumption of 1.32 kW/h and 1.94 kW/h, respectively. In addition to the mechanized milking, Farm 1 also utilized a cooling tank (Sulinox 540L) that consumed 1.70 kW/h. Two daily milking sessions were considered at both properties. Equation 25 was used in the calculation of CO_2e emission, in tons.

After all calculations, the enteric CH_4 emissions data from Trial 1 were used for estimating enteric CH_4 emissions of both farms, observing emissions found for each animal category and the CO_2e total emissions of the two properties were calculated and evaluated regarding their descriptive statistics.

The carbon balance of the evaluated farms was determined using CO_2e emissions data and carbon storage values of the pasture system and of the crops. To calculate storage of the silvopastoral system, a forest census was carried out using an inventory (in March 2013) in which all trees had their DBH (diameter with bark at 1.30 m) and their total and commercial heights measured. The volume was estimated as proposed by Müller et al. (2010). The bole biomass was calculated by using the equation adjusted by Silva et al. (2008). The equations proposed by Paixão (2004) for the biomass of the Zona da Mata biome of Minas Gerais, Brazil, were utilized for the calculation of the biomass of branches and leaves, total carbon, carbon from the organic mass, and carbon from the root mass.

The average annual carbon increment (AACI) of the eucalyptus individuals present in the silvopastoral system was calculated by dividing the estimated amount of total carbon of each individual by their age.

The carbon stock of crops was calculated based on fixation factors determined by

other authors under similar conditions to those of the studied farms (Table 3). Based on the result of the carbon stock calculation, the ton of carbon was transformed into t CO_2 by Equation 26 (see Appendix).

Carbon balance was calculated as the difference between the carbon stock of cultures and pastures, and emissions of CO_2e originating from the dairy activity during the period (CH_4 emissions from enteric fermentation and animal wastes, N_2O emissions from N fertilization and CO_2 emissions from oil used on machinery and electricity consumption with irrigation of crops and with mechanized milking accounted in the calculation).

3. Results

3.1 Trial 1

The mean data of the animals used in Trial 1 are described in Table 4, and the CH_4 emissions obtained from the SF_6 technique compared with those estimated by the IPCC (2006) are shown in Figure 1. Methane emission estimates in g/d were higher for the category of prepubertal heifers when estimated by the equations proposed by the IPCC (2006) ($P < 0.001$). However, the categories of medium-producing, high-producing and dry cows had higher CH_4 emissions ($P < 0.013$) when estimated by the SF_6 technique. Pubertal and pregnant heifers and low-producing cows had similar CH_4 emissions when estimated by the two methods ($P > 0.05$).

Similar results were found when CH_4 emissions were analysed relative to the DMI. However, when evaluated in relation to their productive factors (milk production or average daily gain), CH_4 emissions estimated by the SF_6 tracer gas technique ($P = 0.096$) were less for the low-producing cows and prepubertal heifers.

3.2 Trial 2

The total emissions (enteric CH_4, animal wastes, N fertilization, oil and electrical use) from Farm 1 and Farm 2 were 3.21 t CO_2e/animal/yr and 3.18 t CO_2e/animal/yr, respectively. The majority of CH_4 emissions originated from enteric fermentation, and they were of similar proportions on both farms (Table 5).

Emissions of CO_2e from N_2O on Farms 1 and 2 were 0.49 and 0.64 t CO_2e/animal/yr, respectively (Table 6). A total of 92% of the emissions from N_2O were due to the N fertilization of the pasture (0.453 t) on Farm 1 and 94% on Farm 2.

Emissions of CO_2e from oil use were higher for Farm 2 than Farm 1, 0.127 and 0.083 t CO_2e/animal/yr, respectively (Table 7). On the other hand, CO_2e emissions by electricity were higher for Farm 1 than Farm 2, 0.215 and 0.007 CO_2e/animal/yr (Table 8). These differences result from the intensification of the production system of which Farm 1 emits less CO_2e derived from oil by animal, because the use of oils is diluted by the number of animals but emits more on electricity due to more intensified operations such as irrigation and mechanized milking. The CO_2e from different emission sources and from both farms in CO_2e/animal/yr, CO_2e/L of milk and CO_2e/ha are shown graphically in Figures 2, 3, and 4, respectively.

The carbon balance data obtained with and without carbon stock of pasture and other crops from the two evaluated farms could be estimated by considering enteric CH_4 estimated by the IPCC (2006) or by the SF_6 technique (Fig. 5). Considering Farm 1 and the IPCC (2006) equations, a total balance of 1,441.7 t CO_2e/yr was estimated with soil carbon, and a total balance of -363.5 t CO_2e/yr was estimated without soil carbon. By the SF_6 technique,

however, a total balance of 1,413.1 t CO_2e/yr was estimated with soil carbon, and a total balance of -392.1 t CO_2e/yr was estimated without soil carbon. For property 2, a balance of 162.8 t CO_2e/yr was estimated by the IPCC (2006) considering soil carbon and -25.10 t CO_2e/yr without soil carbon. For the same property, a total balance of 154.00 t CO_2e/yr was estimated using SF_6 with soil carbon and -33.9 t CO_2e/yr without soil carbon.

4. Discussion

4.1 Trial 1

Large differences in estimated (IPCC) and calculated (SF_6) CH_4 emissions were observed. The IPCC (2006) utilizes the equations proposed by the NRC (2001) to calculate gross energy intake. These equations, in the case of heifers, are based on the data of Angus breed animals that are utilized in beef production, which may result in incorrect estimates of intake, especially by heifers with a lower body weight (Hoffman et al., 2008). Angus animals are finished weighing around 450 kg; therefore, 200 kg of body weight represents 44.4% of the adult weight. On the other hand, the adult weight for the Holstein breed is around 650 kg and, therefore, 200 kg represents only 30.7% of their adult weight. Thus, the physiological states of both breeds at 200 kg are completely different, which leads to discrepancies regarding gain efficiency and protein and fat deposition.

Thus, the nutritional requirements of Angus animals may be higher than those of Holstein heifers of the same body weight. The intake of the Holstein heifers will be overestimated by these equations resulting in a similar overestimation of the CH_4 emissions calculated by the IPCC (2006) equations. For the other two categories, pubertal and pregnant heifers, there was no difference between calculated and estimated values (Figure 1). The CH_4

emissions estimated by the two methods follow the same pattern when expressed relative to DMI or weight gain.

For the cows categories, the CH_4 estimates were higher for medium- and high-producing and dry animals based on the SF_6 technique, as compared with those obtained using the IPCC (2006) guidelines. Because IPCC (2006) equations were obtained from animals fed highly digestible diets and animals with a high potential of conversion of feed into animal product, it is possible that these equations underestimate the CH_4 emission potential of the animals when they are fed forage of a lower nutritional value (digestibility) (Burns and Fisher, 1991). By the same reasoning, the CH_4 emissions obtained by the IPCC (2006) equations were also underestimated for high- and medium-producing and dry cows when expressed relative to DMI values. This result may, likewise, stem from the quality of the forage utilized in temperate countries, where the IPCC (2006) equations were generated, even if the necessary corrections are made.

In addition to negatively affecting intake, the lower digestibility of the forage is linked to a higher proportion of fibrous material and greater acetate production during the ruminal fermentation process. This generates a larger amount of H_2 and CO_2 ions in the rumen environment as end products of this process, which will be utilized in methanogenesis (Hungate, 1966; Knapp et al., 2014).

When expressed as a function of milk yield, CH_4 emissions by the IPCC (2006) were underestimated for the medium- and high-producing cows, and there was an overestimation trend for the low-producing cows.

4.2 Trial 2

It was found that much of the CH_4 emissions by dairy cattle (67.08% Farm 1 and 71.43% Farm 2) are related to the digestive process of ruminants, indicating that enteric CH_4 is a key factor for mitigation actions. The annual milk production may account for some of the difference between the CH_4 emissions of the herds from both properties. While adult dairy cattle from the intensive property (Farm 1) required 60.88 MJ/d NE_L to produce 22.27 L of milk with 3.16% fat, the animals from the semi-intensive property (Farm 2) required 47.82 MJ/d NE_L to produce, on average, 10.5 L of milk with 4.57% fat.

The CH_4 emissions from herds on both farms were consistent with data indicated by the Brazilian Ministry of Science and Technology (MCT, 2006) for dairy cattle (70 kg CH_4/animal/yr). On the other hand, the emissions associated with animal wastes (0.83 t CO_2e/animal/yr) was lower than that suggested by MCT (2006) for dairy cattle (1.8 t CO_2e/animal/yr) and beef cattle (1.5 t CO_2e/animal/yr).

The MCT (2006) data represent an average of the estimated total number of animals in the region, and it is thus subjected to variations according to cattle management. Even though the MCT (2006) considers a semi-intensive system for their emissions calculations (i.e., the animals are raised on pasture and concentrate, with forage supplementation provided only at the time of lowest forage growth, and the average feed intake is 7.9 kg), the estimate appears to be accurate for the farms evaluated in this study.

For the other emissions, the highest value was associated with pasture fertilization on Farms 1 and 2, and it is due to greater nutritional requirement compared to the other crops and to additional emissions caused by the incorporation of animal wastes. According to Cantarutti and Santos (2002), the animal interferes with the amount of nutrients removed from the pasture, and this depends on the type of exploitation and intensity of the production system.

The CH_4 emissions resulting from enteric fermentation and animal wastes were the most important on both farms. Despite the fact that other emission sources contribute to increasing the total emissions by dairy livestock, they do not represent a major concern of the sector. Because wastes were deposited on pasture as organic fertilizer, the values from animal waste emissions were considered low when compared with the use of anaerobic storage conditions. According to Williams (1993), in studies conducted in Australia, CH_4 production from faeces is 3% than that produced in the rumen. Only the management with anaerobic lagoons would be capable of producing significant emissions of CH_4 from beef- and dairy-cattle faeces (Lodman et al.,1993).

Even on Farm 1, where the technological level is higher, the application of N fertilization, utilization of fossil fuels, and electrical energy are small contributors to the total emissions from the dairy livestock activity. Nitrogen fertilization displayed the greatest emission potential from the indirect emissions. The emissions from N fertilization demonstrate that the intensification of a production system results in lower N_2O emissions per unit of product (Kebreab et al., 2001). This is consistent with the results of the present study showing an emission of 0.132 t CO_2e/L in the more intensive system and 0.356 t CO_2e/L in the semi-intensive system. In addition, the greater emissions from N fertilization over the emissions from oil use and electricity consumption is mostly due to the global-warming potential of N_2O, which is 298 times higher than an equal mass of CO_2 (Prather et al., 2001).

The results obtained for carbon balance in this study are consistent with the results from a case study in New Zealand, in which Handford et al. (2011) observed a total emission of 3.55 t CO_2e/animal and also concluded that the livestock (86%) are a major source of total emissions. In spite of that, Capper et al. (2009) evaluated databases from the year 2007 to

determine the emissions from farms located in the United States. They found emissions of 0.03 t CO_2e/animal and 0.00135 t CO_2e/L of milk. These differences reinforce the need for the development of models which can better represent the peculiarities of each country and might be due to differences in how soil carbon was estimated.

In studies compiled by Conant et al. (2001), the mean carbon sequestration rates varied from −0.2 to +3.0 Mg C ha/yr, considering a well-managed pasture. According to Braga (2010), the analysis of carbon accumulation in the soil can take several years and, therefore, a year is considered a short time. We know that carbon soil makes the reduction targets easier to achieve (Byrne et al., 2007). Thus, we suggest that future research should focus on changes in soil carbon over several years, in order to accurately estimate carbon sequestration.

Therefore, the carbon balance was calculated considering the biomass found above the soil. In order to estimate pasture carbon and other crops interference in the carbon balance, we calculated the pasture carbon stock, which can be considered representative due to a few modifications that the amount of carbon in the culture may suffer throughout the year. Nevertheless, we recognize that the use of carbon sequestration is important in carbon emission estimates because it helps to offset GHG emissions, since it is a great mitigation mechanism for agriculture (Soussana et al., 2009).

Carbon balance estimates considering pasture carbon and other crops suggests that both farms are sustainable, since the carbon in soil is high enough to overcome the emission of the systems, by both methodologies. Therefore, the total emissions are offset by the carbon in the pasture and other crops. On the other hand, when this stock is not in the equation for carbon balance, the estimates suggest that both farms emit more carbon than are capable of stock in the soil.

The difference on the total equivalent carbon balances considering the two methods evaluated was only 1.98% on Farm 1 and 5.42% on Farm 2. Of the differences on CH_4 emissions found between the two methods (Figure 1), the SF_6 technique produced higher estimates in some categories, whereas the IPCC (2006) equations generated higher estimates for other categories. This fact offset the total equivalent carbon estimates from the properties, resulting in a similar carbon balance on the two farms, regardless of the method. Nevertheless, it was noted that the largest differences in the estimates were observed between the medium- and high-producing cows.

The methodology recommended by the IPCC (2006) should be used with care in tropical conditions. It can over- or underestimate CH_4 emissions from different animal's categories. However, in the final balance of the whole farm, the inconsistences are nullified, when considering a herd compound of different categories. Thus, it can be observed that the composition of the herd is an important variable to be evaluated for taking the decision between IPCC (2006) calculations and SF_6 technique to estimate CH_4 emissions. In the current scenario, in which Brazil has most of its dairy animals producing less than 20 kg/d, IPCC (2006) equations seems to be adequate to estimate the carbon balance of the properties. However, the future scenario is a cause for concern because Brazil has consistently increased its total production and its production per animal (IBGE, 2012; Maia et al., 2013). Therefore, it is possible in the near future, that the IPCC (2006) equations will underestimate the enteric CH_4 emissions and overestimate the carbon balance of dairy farms, given that high- and medium-producing cows together account for 25.6 and 83.8% of the total enteric CH_4 production, and 13.1 and 44.7% of the total equivalent carbon emission from farms 1 and 2, respectively.

5. Conclusions

IPCC (2006) equations should be used with care in tropical conditions since CH_4 emissions estimated by this method showed some discrepancies when considering individual categories, underestimating CH_4 emissions from medium-, high-producing and dry cows and overestimating CH_4 produced by 200-kg heifers. However, the predicted values obtained for heifers from 350 kg and 450 kg and for low-producing are, on average, similar to those obtained by the SF_6 technique. Because of an offset in the discrepancies found in the present study, when considering the whole farm emissions, the equations suggested by the IPCC (2006) correctly estimate the total CH_4 emission and carbon balance on small dairy farms of Minas Gerais State, Brazil. However, the decision between IPCC (2006) calculations and SF_6 technique to estimate CH_4 emissions from each farm should consider the composition of the herd.

Further research similar to the present study should focus on the analysis of the carbon stock and sequestration in soil, pasture and crops to achieve a more accurate estimate of carbon balance and, therefore, to encourage mitigation actions and programs by the producers in association with companies and with the Government.

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7. References

Amado, T.J.C., Bayerc, F.L.F. and Brum, A.C.R., 2001. Potential of cover crops to sequester carbon and increase soil nitrogen content, under no-tillage system, improving environmental quality. R. Brasi. Ci. Solo, 25, 189-197.

Association of Official Analytical Chemists (AOAC) 1990 Official Methods of Analyses of the AOAC, pp. 1–1230, 15 edition. Washington: Assoc OFF Agric Chemp.

Bernd A., and Tomkins, N.W., 2013. Measurement and mitigation of methane emissions from beef cattle in tropical grazing systems: a perspective from Australia and Brazil. Animal, 7:s.2, pp. 363-372.

Braga, G.J., 2010. Carbon sequestration in cultivated pastures. Pesquisa & Tecnologia, 7. Available in http://www.aptaregional.sp.gov.br/acesse-os-artigos-pesquisa-e-tecnologia/edicao-2010/2010-janeiro-junho/765-sequeestro-de-carbono-em-pastagens-cultivadas/file.html. Accessed 07/20/2015.

Brasil, 2006. Ministério da Ciência e Tecnologia. Emissões de dióxido de carbono e de metano pelos reservatórios hidrelétricos brasileiros: relatórios de referência: primeiro inventário brasileiro de emissões antrópicas de gases de efeito estufa. Brasília: MCT, 2006. 118 p.

Brasil, 2007. Contagem populacional IBGE: Ministério do Planejamento, Orçamento e Gestão. Rio de Janeiro, 2007.

Burns, J.C., Fisher, D.S., 1991. Tropical corn for silage in multiple cropping systems. In The environment and tropical corn. Proc. Southern Regional Tropical Corn Symposium, Potash and Phosphate Institute, Atlanta, GA, pp 37 41. ID 1151. Available in www.internationalgrasslands.org/files/igc/publications/1997/2-19-043.pdf. Accessed 07/01/2015.

Byrne, K.A., Kiely, G. and Leahy, P., 2007. Carbon sequestration determined using farm scale carbon balance and eddy covariance. Agric. Ecosyst. and Environ. 121, 357–364.

Campos, D.C., 2003. Carbon sequestration potenciality of sugarcane harvesting without burning. Ph.D. Thesis, Universidade de São de Paulo.

Cantarutti, R.B. and Santos, H.Q., 2002. Nutrient entry and conservation on pasture based beef cattle systems. In: Figueiredo F. C., Magalhães K. A., Marcondes M. I., Chizzotti M. L., Guimarães J. D., Paulino M. F. and Valadares Filho S. C. (eds.) III Symposium

of Beef Cattle Production, pp. 133-152. Viçosa-MG: UFV.

Caper, J. L., Cady, R. A., Bauman, D. E., 2009. The environmental impact of dairy production: 1944 compared with 2007. J. *Anim. Sci.* 87, pp. 2160-2167.

Cederberg, G.C and Mattson, B., 2000. Life cycle assessment of milk production - a comparison of conventional and organic farming. J. Clean. Production, 8, 49-60.

Conant R.T., Paustian, K. and Elliot, E.T., 2001. Grassland management and conversion into grassland: Effects on soil carbon. Ecol. Appl. 11, 343-355.

Goes, A.L.M., Freitas, I.M., Rangel, J.H.A., Muniz, E.M. and Santos, D.O., 2013. Elephant grass genotypes evaluation for biomass energy production in the state of Sergipe. Proceedings of the III Graduate Scientific Seminar of Embrapa Tabuleiros Costeiros, Aracaju-Brazil, pp. 183-190.

Hoffman, P.C., Weigel, K.A., Wernberg, R.M., 2008. Evaluation of equations to predict dry matter intake of dairy heifers. J. Dairy Sci. 91, 9, 3699-3709.

Handford, P.A. et al., 2011. Carbon balance – Dairy farm. Farming Carbon in New Zealand: info sheets 9 (version 3). Available in http://www.carbonfarming.org.nz/wp-content/uploads/InfoSheet_9v3b.pdf. Accessed 07/10/2015.

Hungate, R.E., 1966. The rumen and its microbes. Academic Press. New York, NY.

IDF (International Dairy Federation). (2010). A common carbon footprint approach for dairy. The IDF guide to standard lifecycle assessment methodology for the dairy sector. *Bulletin of the International Dairy Federation* 445/2010. IDF, Brussels, Belgium.

IBGE (2012). Produção da pecuária municipal. Rio de Janeiro, V. 40, P.1-71

IPCC (2006) Intergovernmental panel on climate change: Emissions from livestock and manure management. In: EGGLESTON S., BUENDIA L., MIWA K., NGARA T. AND TANABE K. (eds.) IPCC Guidelines for National Greenhouse Gas Inventories, pp. 10.1-10.84. Hayama, Japan: Institute for Global Environmental Strategies.

Johnson, K.A., Huyler, M., Westberg, H.H., Lamb, B. and Zimmerman, P., 1994. Measurement of methane emissions from ruminant livestock using a sulphur hexafluoride tracer technique. Environ. Sci. Technol. 28:359–362.

Johnson, K.A., Westberg, H. H., Michal, J. J. and Cossalman, M.W., 2007. The SF_6 tracer technique: Methane measurement from ruminants. In: Makkar H. S. and Vercoe P. (eds.) Measuring methane production from ruminants, pp. 33-67: Springer Netherlands.

Knapp, J.R., Laur, G.L., Vada, P.A., Weiss, W.P., Tricarico, J.M., 2014. Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions, J. Dairy Sci. 97, pp. 3231-3261

Kurihara, M., Magner, T., Hunter, R. A., McCrabb, G. J., 1999. Methane production and energy partition of cattle in the tropics. Br. J. Nutr. 81, n.3, p.227-234.

Kebreab, E., France, J., Beever, D.E. and Castillo, A.R., 2001. Nitrogen pollution by dairy cows and its mitigation by dietary manipulation. Nutr. Cycling Agroecosyst. 60, 275-285.

Lima, M.A., Pessoa, M.C.P.Y. and Ligo, M.A.V., 2002. Livestock methane emission: Reference reports. Brasília, BR: MCT - Ministério de Ciência e Tecnologia.

Lodman, D.W., Branine, M. E., Carmean, B.R., Zimmerman, P., Ward, G.M. and Johson, D.E., 1993. Estimates of methane emissions from manure of U.S. Cattle. Chemosphere, 26, 189-199.

Maia, G.B.S, Pinto, A.R, Marques, C.Y.T., Roitman, F.B., Lyra, D.D., 2013. Produção Leiteira no Brasil. Agropecuária, BNDS Setorial, 37, p.371-398.

Mello, S.L.D.M. and Gonçalves, J.L.D.M., 2008. Equations to estimate shoot and root system biomass in eucalyptus grandis stands at sites with different productivities. Revista Árvore, 32, 101-111.

Müller, M.D., Fernandes, E.N., Castro, C.R.T., Paciullo, D.S.C. and ALVES, F.F., 2010. Estimate of biomass and carbon storage by an agrossilvipastoral system in the zona da mata mineira. Pesquisa Florestal Brasileira, 11.

National Research Council - NRC. Nutrient requirements of dairy cattle. 7.rev.ed. Washinton, D.C.: 2001. 381p.

Paixão, F.A., 2004. Carbon inventory in eucalyptus forest and economic evaluation of

different management alternatives Masters in Forestry Engineering Dissertation, Universidade Federal de Viçosa.

Prather, M., Ehhalt, D., et al., 2001. Atmospheric chemistry and green-house gases, In: Climate Change 2001: The Scientific Basis, edited by: Houghton, J. T., Ding, Y., Griggs, D. J., et al., 239–287, Cambridge University Press, Cambridge, UK.

Primavesi, O., Frighetto, R.T.S., Pedreira, M.S., Lima, M.A., Berchielli, T.T., Demarchi, J.J.A.A., Manella, M.Q., Barbosa, P.F., Johnson, K.A. and Westberg, H.H., 2004. Técnica do gás traçador sf6 para medição de campo do metano ruminal em bovinos: Adaptações para o Brasil, p. 76. São Carlos - SP: Embrapa.

Santos, D.R.R., Picanço, A.P., Maciel, G.F. and Serra, J.C.V., 2010. Study of greenhouse gases offsets of the Federal University of Tocantins State, Brazil – academic senate and campus of the University of Palmas: A way of environmental mitigation. Revista Geográfica Acadêmica, 4, 29-40.

Silva, R.F.D., Soares, C.P.B., Jacovine, L.A.G., Silva, M.L.D., Leite, H.G. and Silva, G.F.D., 2008. Carbon stock projection and credit generation analysis in eucalypt stands. Revista Árvore, 32, 979-992.

Soussana, J.F., Tallec, T., Blanfort, V., 2009. Mitigating the greenhouse gas balance of ruminant production systems through carbon sequestration in grasslands. Animal. 4:3, pp. 334-350.

United Nations 1998. Kyoto protocol to the united nations framework convention on climate change, p. 21. New York, US: United Nations.

Williams, D.J., 1993. Methane emissions from manure of free-range dairy cows. Chemosphere, 26, 179-187.

Zen, S., Barioni, L.G., Bonato, D.B.B., Almeida, M.H.S.P. and Rittl, T.F., 2008. Brazilian livestock: Environmental impacts and emissions of greenhouse gases (GHG) Offical Government Report, pp. 1-6. São Paulo: CEPEA.

8. Appendix

List of equations and description of the meaning of each term.

$$E_{ferm} = (E_{milk} \times P)/10^3 \quad [1]$$

E_{ferm}	total CH_4 emission from enteric fermentation (t CH_4/yr)
E_{milk}	emission factor for milk production (kg CH_4/animal/yr)
P	total number of animals in the herd

$$E_{milk} = (GE_{milk} \times Y_m \times P)10^3 \quad [2]$$

GE_{milk}	gross energy intake by the animals (MJ/animal/d)
Y_m	CH_4 conversion rate (0.14)

$$GE_{milk} = [((NE_m + NE_f + NE_L + NE_p)/RND) + (Ne_gRND_g)] \times 100/D \quad [3]$$

NE_m	net requirements for maintenance (MJ/animal/d)
NE_f	net energy for feeding activities (MJ/animal/d)
NE_L	net energy for lactation (MJ/animal/d)
NE_g	net energy for growth (MJ/animal/d)
NE_p	net energy for pregnancy (MJ/animal/d)
RND	ratio between energy consumption for maintenance, feeding activities, lactation, pregnancy and digestible energy intake

RND_g ratio between energy intake gain and digestible energy intake

D total digestibility

$$NE_m = 0.335 \times W^{0.75} \quad [4]$$

W animal weight (kg)

$$NE_f = NE_m \times 0.17 \quad [5]$$

$$NE_L = MP \times (1.47 + 0.40 \times MF) \quad [6]$$

MP milk production (kg/animal/d)

MF milk fat (%)

$$NE_p = 0.075 \times NE_m \times PR/100 \quad [7]$$

PR pregnancy rate (%)

$$NE_g = 4.18 \times [(0.035 \times W^{0.75} \times WG^{1.119}) + WG] \quad [8]$$

WG average weight gain (kg/d)

$$RND = 0.298 + (0.00335 \times D) \quad [9]$$

$$RND_g = -0.036 + (0.00535 \times D) \quad [10]$$

$$E_{waste} = (EF_{waste} \times P)/10^3 \quad [11]$$

E_{waste} CH_4 emission from animal wastes (t CH_4/yr)

EF_{waste} waste emission factor from dairy animals (kg CH_4/animal year)

$$EF_{waste} = VS \times B_o \times \frac{MCF \times FA}{10^4} \times 365 \times 0.67 \quad [12]$$

VS daily excretion of volatile solids from dairy animals (kg/animal/d)

B_o CH_4 conversion factor for the waste type (% of waste dry matter) (%)

FA FA = fraction of animals linked to adopted waste management (%)

$$VS = GE \times (1/18.45) \times ((1 - D/100) \times (1 - ASH/100)) \quad [13]$$

ASH ash percentage in wastes (%)

$$E_{N_2O} = E_{N_2O_d} + E_{N_2O_i} + E_{N_2O_p} \quad [14]$$

E_N2_O total N_2O emission (t N_2O/ha/yr)

E_N2_Od total direct N_2O emission (t N_2O/ha/yr)

E_N2_Oi total indirect N_2O emission (t N_2O/ha/yr)

E_N2_Op total pasture N_2O emission from animal wastes (t N_2O/ha/yr)

$$E_{N_2O_d} = (F_{SN} + F_{AW}) \times EF_1 \quad [15]$$

F_{SN} N applied in the soil from synthetic fertilizers, corrected for NH_3 and NO_x emissions (kg N/yr)

F_{AW} FAW = manure spread daily on the soil as fertilizer, corrected for NH_3 and NO_x emissions (kg N/yr)

EF_1 factor of direct emission of N applied in the soil (kg N_2O-N/kg N = 0.0125)

$$F_{SN} = N_{fert} \times (1 - Frac_{GASF}) \quad [16]$$

N_{fert} amount of N applied as synthetic fertilizer (kg N/yr)

$Frac_{GASF}$ amount of N applied as synthetic fertilizer that volatizes as NH_3 and NO_x ([kg NH_3-N and NO_x-N]/kg N_2O-N applied = 0.10)

$$F_{AW} = N_{manure} \times (1 - Frac_{GASM}) \quad [17]$$

N_{manure} total N from manure applied in the soil (kg N/yr)

$Frac_{GASM}$ amount of N excreted by animals that volatizes as NH_3 and NO_x ([kg NH_3-N and NO_x-N]/kg N_2O-N applied = 0.20)

$$N_{manure} = N_{exL} \times Frac_{milk} \quad [18]$$

N_{exL} total N excreted by animals (kg/yr)

$Frac_{milk}$ fraction of manure applied as fertilizer (1)

$$N_{exL} = P \times N_{milk} \quad [19]$$

N_{milk} total N excreted per animal (kg N/animal/yr)

$$E_{N_2O_i} = N_2O_G + N_2O_L \quad [20]$$

N_2O_G N_2O produced from atmospheric deposition of NH_3 and NO_x (N_2O-N/yr)

N_2O_L N_2O produced from leaching and surface flow of N (N_2O-N/yr)

$$E_{N_2O_G} = [(N_{fert} \times Frac_{GASF}) \times (N_{manure} \times N_{pasture}) \times Frac_{GASM}] \times EF_4 \quad [21]$$

$N_{pasture}$ N applied in the pasture from animal waste (kg N/yr)

EF_4 emission factor for atmospheric deposition (kg N_2O-N/[kg NH_3-N and NO_x-N emitted] = 0.01)

$$E_{N_2O_L} = [(N_{fert} + N_{manure} + N_{pasture}) \times Frac_{leach}] \times EF_5 \quad [22]$$

$Frac_{leach}$ Fraction of N applied to soil lost by leaching or surface flow (kg of N leached or flowed/kg of fertilizer = 0.3)

EF_5 N_2O emission factor for leaching or surface flow (kg N_2O-N/kg N leached or flowed = 0.025)

$$N_2O_{pasture} = P \times N_{exL} \times AWMS_{pasture} \times EF_3 \quad [23]$$

$AWMS_{pasture}$ fraction of animal-excreted N in the pasture

EF_3 N_2O emission factor for the evaluated system (grassland management) (kg N_2O-N/kg N excreted = 0.02)

$$E = \frac{\sum C_i \times FE_i}{1,000} \quad [24]$$

E emission of carbon equivalent (t CO_2eq)

C consumption of gas *i* (L)

FE_i factor of CO_2 emission associated with gas *i* (kg CO_2/L)

$$E = \sum CE_m \times FE_m \quad [25]$$

CE total energy consumed per month (MWh)

FE energy emission factor in the month (t CO_2eq/MWh)

$$tCO_2eq = \frac{\left(\frac{C * 44}{12}\right)}{1000} \quad [26]$$

t CO_2eq carbon dioxide equivalent, in tons

C carbon content, in kg

44/12 specific mass of carbon dioxide (44) on the specific mass of the element carbon (12)

Table 1

Description of forage:concentrate ratios of each animal category.

Categories	Corn silage	Concentrate	Coastcross grass hay
Heifers	90.00	10.00	-
High-producing cows	41.50	50.00	8.50
Medium-producing cows	70.00	30.00	-
Low-producing cows	80.00	20.00	-
Dry cows	90.00	10.00	-

Table 2

Description of data of the evaluated systems.

Item	Unit	Farm 1	Farm 2
Herd	Animals	113.0	12.0
Pasture area	ha	38.5	1.0
Dairy area	ha	53.0	7.8
Total area	ha	202.0	15.6
Annual milk production	L/yr	420,143	37,011
Average digestibility (D)	%	70.0	70.0
Average weight (W)	kg	550.0	570.0
Weight gain (WG)	kg/animal/d	0.3	0.5
Pregnancy (PR)	%	25.0	60.0
Milk fat (MF)	%	3.2	4.6

Table 3

Carbon fixation factor in tropical conditions for the following crops.

Crops	Factor (t C ha/yr)	Reference
Pastures	0.60	Braga (2010)
Sugarcane	1.50	Campos (2003)
Elephant grass production for cut	18.6	Goes et al. (2013)
Corn	2.78	Amado et al. (2001)
Eucalyptus	14.51	Mello and Gonçalves (2008)

Table 4

Descriptive data of animals used in CH_4 determination by the SF_6 technique.

Item	High-producing cows	Medium-producing cows	Low-producing cows	Dry cows	Prepubertal heifers	Pubertal heifers	Pregnant heifers	SEM
Live weight	453	611	610	746	200	359	473	27.86
Dry matter intake (kg/d)	15.08	17.37	13.13	11.16	4.43	7.13	7.04	0.88
Milk production (L/d)	27.09	15.54	10.14	-	-	-	-	1.94
Average daily gain (kg/d)	-	-	-	-	1.15	1.67	0.91	0.12
Mean age	3 yr	4 yr	3 yr	5 years	9 mo	19 mo	24 mo	-

Table 5

Methane emissions from enteric fermentation and animal wastes from dairy cattle in two Brazilian systems.

Category	Emission				
	Per animal	Per animal	Proportion	Per litre of milk	Per ha
	kgCH_4/animal/yr	t CO_2e/animal/yr	%	kg CO_2e/L	t CO_2e/ha
Farm 1 (intensive production)					
Enteric CH_4	68.10	1.63	67.08	0.44	3.48
Animal wastes	33.23	0.80	32.92	0.21	1.71
Total	101.33	2.43	-	0.65	5.18
Farm 2 (semi-intensive production)					
Enteric CH_4	70.78	1.70	71.43	0.55	2.62
Animal wastes	28.42	0.68	28.57	0.22	1.05
Total	99.20	2.38	-	0.77	3.66

Table 6

Emission of direct, indirect, and N_2O from animal waste from productive areas of two Brazilian dairy systems.

Category	Emission					
	Per area			Per animal	Per litre of milk	Per ha
	t ha/yr			t CO_2e/animal/yr	kg CO_2e/L	t CO_2e/ha
	Direct N_2O	Indirect N_2O	Pasture N_2O	CO_2eq		
Farm 1 (intensive production)						
Pasture	9.01×10^{-2}	6.10×10^{-3}	7.62×10^{-2}	0.453	0.122	0.966
Corn	4.00×10^{-3}	5.07×10^{-3}	-	0.024	0.006	0.050
Sugarcane	6.63×10^{-4}	2.55×10^{-3}	-	0.009	0.002	0.018
Elephant grass for cutting	4.27×10^{-4}	2.37×10^{-3}	-	0.007	0.002	0.016
Total	**0.095**	**0.016**	**0.076**	**0.493**	**0.132**	**1.050**
Farm 2 (semi-intensive production)						
Pasture	1.19×10^{-2}	4.70×10^{-3}	7.56×10^{-3}	0.601	0.194	0.925
Corn	1.85×10^{-3}	4.43×10^{-3}	–	0.022	0.006	0.034
Sugarcane	4.98×10^{-4}	2.42×10^{-3}	–	0.008	0.002	0.012
Eucalyptus	8.38×10^{-3}	2.36×10^{-3}	–	0.007	0.002	0.011
Total	**0.023**	**0.014**	**0.008**	**0.638**	**0.204**	**0.982**

Table 7

Emission of CO_2e from oil use in two Brazilian dairy systems.

Category	Use	Emission			
		Total	Per animal	Per litre of milk	Per ha
	Litres	t CO_2e/yr	t CO_2e/animal/yr	kg CO_2e/L	t CO_2e/ha
Farm 1 (intensive production)					
Ploughing	878	2.35	0.019	0.005	0.041
Disking	2,637	7.07	0.058	0.016	0.124
Ensiling	287	0.77	0.006	0.002	0.013
Total	**3,802**	**10.19**	**0.083**	**0.023**	**0.177**
Farm 2 (semi-intensive production)					
Ploughing	129	0.34	0.028	0.009	0.043
Disking	388	1.04	0.086	0.028	0.132
Ensiling	56	0.15	0.013	0.004	0.020
Total	**573**	**1.53**	**0.127**	**0.041**	**0.195**

Table 8
Emission of CO_2eq by electricity consumption in two Brazilian dairy systems.

Category	Use	Emission			
		Total	Per animal	Per litre of milk	Per ha
	kWh/yr	t CO_2e/yr	t CO_2e/animal/yr	kg CO_2e/L	t CO_2e/ha
Farm 1 (intensive production)					
Milking parlor	2,832	0.26	0.002	6.04×10^{-4}	0.004
Milk cooler	14,892	1.36	0.011	0.003	0.024
Irrigation	216,240	22.90	0.202	0.047	0.349
Total	**233,964**	**21.43**	**0.215**	**0.051**	**0.377**
Farm 2 (semi-intensive production)					
Milking parlor	964	0.09	0.007	0.002	0.011
Total	**964**	**0.09**	**0.007**	**0.002**	**0.011**

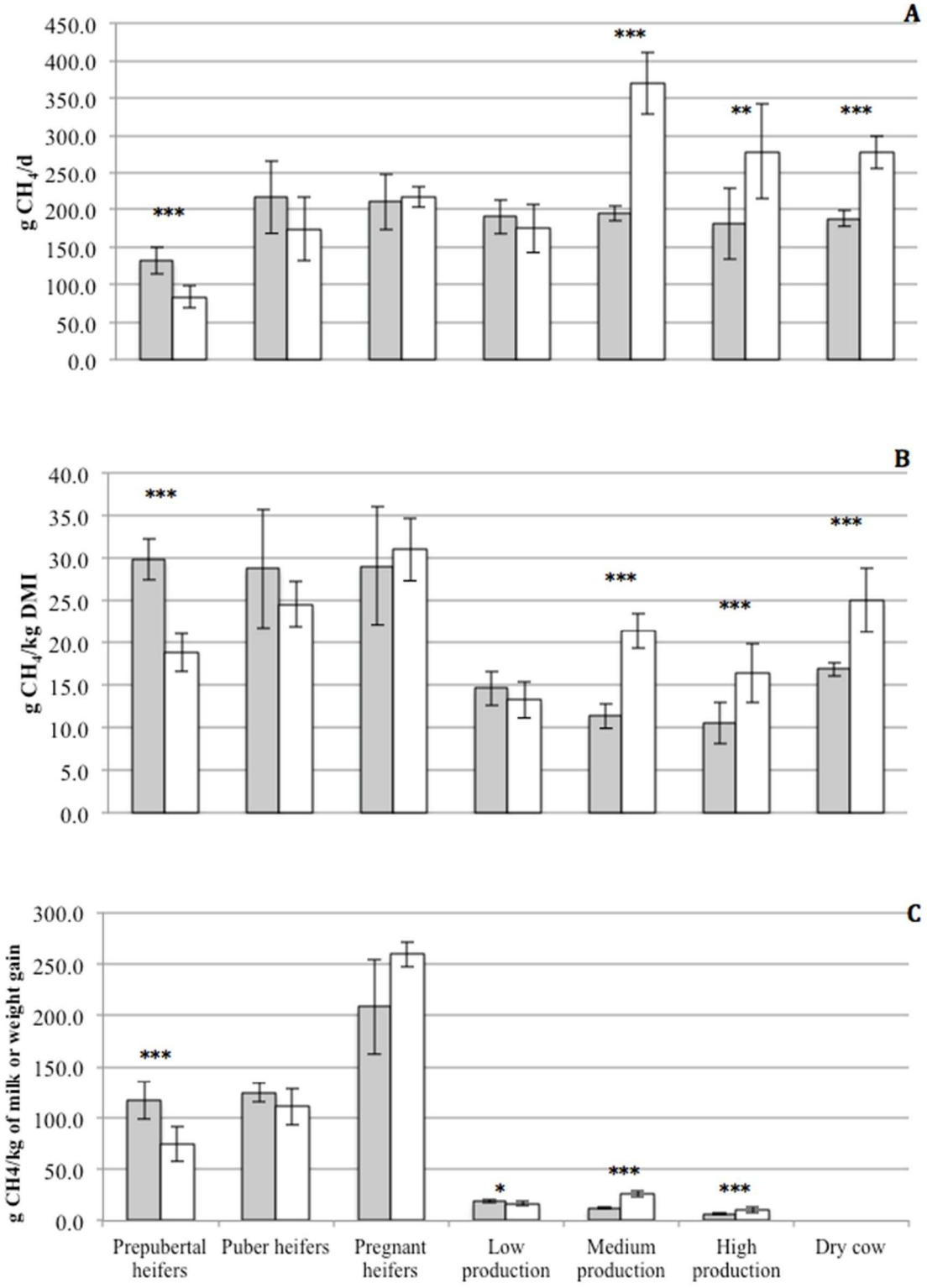

Figure 1**.** Methane emissions estimated by IPCC equations (gray bars) or determined by the SF_6 technique (white bars) in g/day (A), g/kg of DMI (B) and g/kg of milk or weight gain (C). The symbol "*" indicates significance at 0.10, "**" indicates significance at 0.05, and "***" indicates significance

at 0.001.

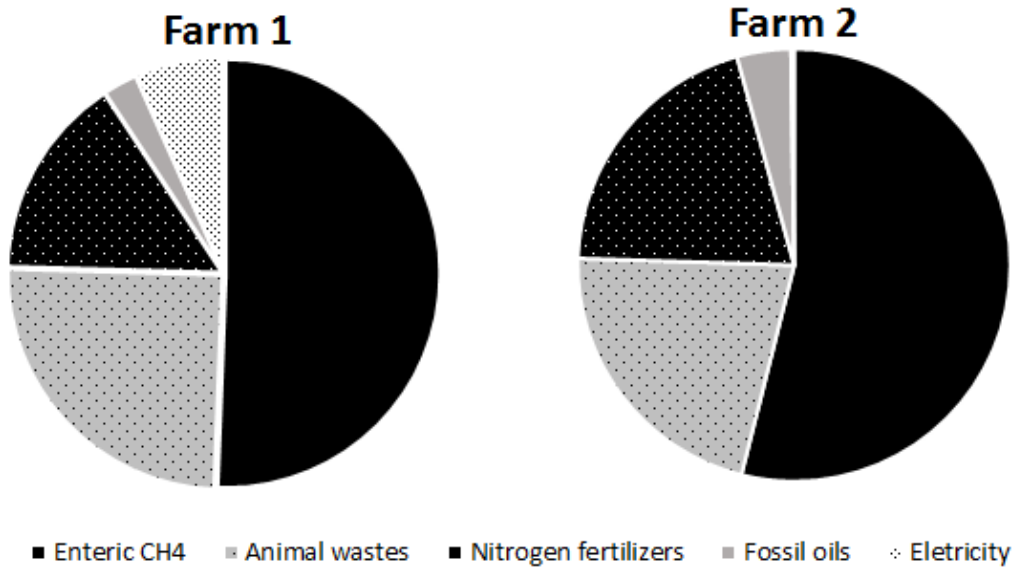

Figure 2. Carbon dioxide equivalent emission, per animal (CO_2e/animal), from different sources in Farms 1 and 2.

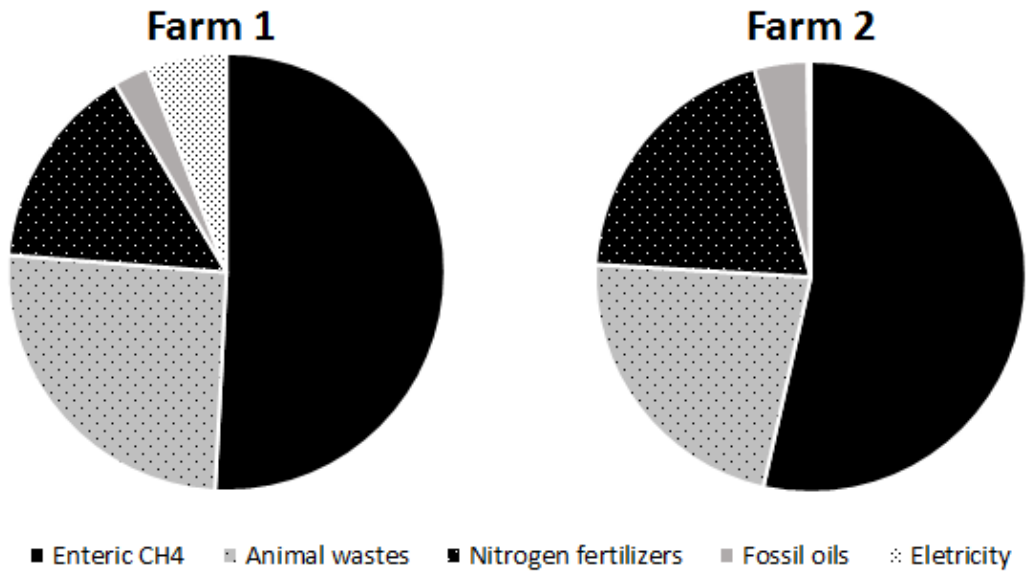

Figure 3. Carbon dioxide equivalent emission, per litre of milk (CO_2e/L), from different sources in Farms 1 and 2.

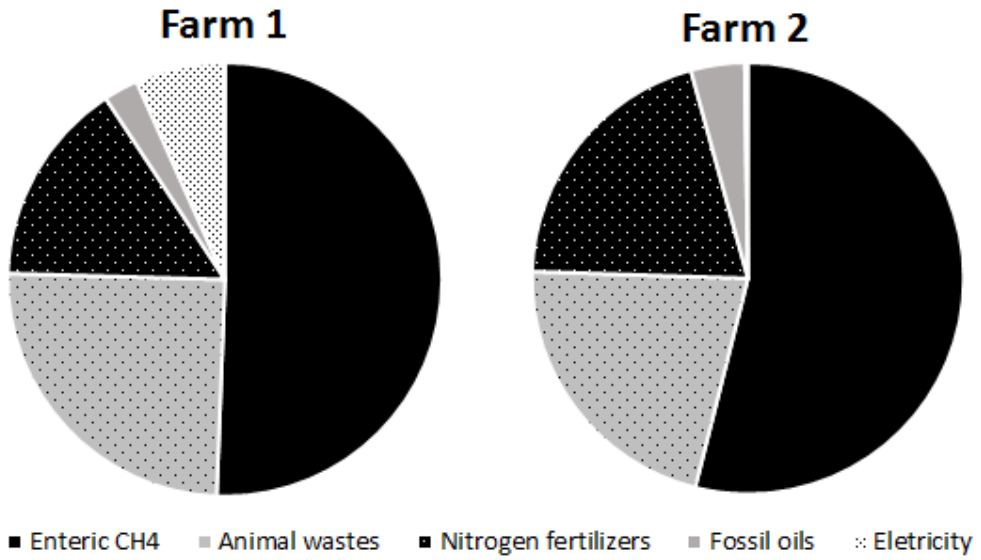

Figure 4. Carbon dioxide equivalent emission, per ha (CO_2e/ha), from different sources from Farms 1 and 2.

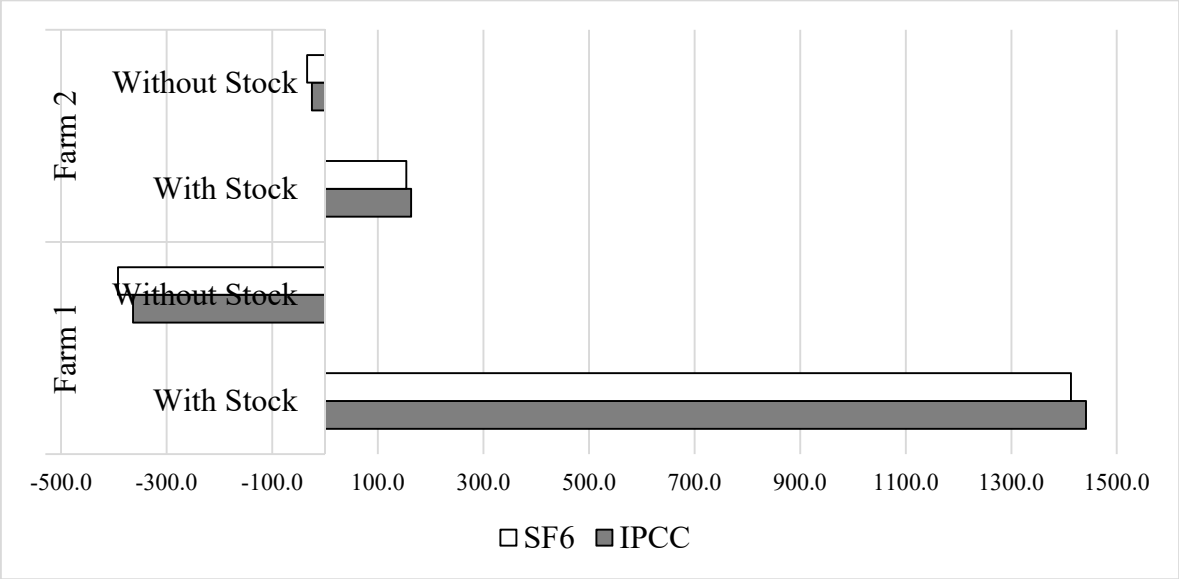

Figure 5. Carbon balance, in tCO_2e/year, from Farm 1 and 2, estimated with and without soil carbon and using enteric CH_4 obtained from IPCC equations and from the SF_6 technique.