MARCELO GROSSI MACHADO

IMPACTS OF DIET INVERSION ON VOLUNTARY INTAKE, DIGESTA AND FECAL COMPOSITION, AND BACTERIAL COMMUNITY COMPOSITION IN RUMEN OF CATTLE FED TROPICAL FORAGE-BASED DIETS

Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fullfillment of the requirements for the degree of Magister Scientiae.

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Approved: December 7, 2015

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BIOGRAPHY

Marcelo Grossi Machado, son of Juarez Guerra Machado and Helenice Cunha Grossi, was born in Barbacena, Minas Gerais, Brazil, on May 21, 1990. He grew up between a countryside living in his parents' hometown Mercês and Barbacena until 2004 when he started to study at the Federal Agrotechnical School of Barbacena, receiving a degree of Animal Scientist Technician in 2007.

After a small period of working on dairy farms on his region, he started the undergraduate course in Animal Science at Universidade Federal de Viçosa in 2008. Between 2011 and 2012 he left Viçosa and moved to Ashburton, New Zealand, where exerted a dairy assistant job for one year. Once back, he got a Bachelor of Science degree in Animal Science in 2013. On October of the same year started the Master's program in the same university with major on nutrition and production of ruminants.

On 7th of December 2015, Marcelo has submitted his dissertation to the thesis committee to obtain the Magister Scientiae degree in Animal Science.

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ABSTRACT

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The objective of this study was to evaluate the impact of the diet inversion on voluntary intake, ruminal digesta and feces composition, ruminal fermentation, and bacterial community composition in cattle fed tropical forage-based diest and to establish the minimal length of the adaptation period that could be applied to changeover or crossover design protocols. Twelve rumen-cannulated crossbreed (B. taurus × B. indicus) bovines were used in this experiment (four young bulls, four steers, and four heifers). The experiment lasted 60 d and two diet inversions were done at d 21 and d 41. Four diet sequences were evaluated (one animal of each category per sequence): sugarcane-corn silage-sugarcane, corn silage-sugarcane-corn silage, high-low-high concentrate diets, and low-high-low concentrate diets. All diets were adjusted to 110 g crude protein per kg of dry matter by using urea. Four group of variables were evaluated along experimental days: voluntary intake characteristics, fecal excretion and composition, ruminal digesta compostion, and ruminal fermentation profile. The pattern of the groups along time was interpreted through a non-hierarchical clustering procedure. The diversity of the liquid-associated rumen bacterial community was evaluated by PCR-DGGE in the young bulls from 6 days before to 20 days after the second diet inversion. The pattern of microbial diversity was evaluated through a clustering procedure using the unweight pair group method with arithmetic mean. Stabilization of the voluntary intake, ruminal digesta, fecal excretion and composition, and rumen fermentation profile was achieved within 9-13, 9-14, 6-13, and 4-11 days after diet inversion, respectively. The bacterial community in the liquid phase stabilized within 3-9 days after diet inversion. The maximum required adaptation period among all characteristics must be chosen to assure the utilization of a robust experimental protocol. From the results of this experiment, among all obtained times to adapt intake, fecal and digesta composition, and ruminal fermentation and microbial diversity, a 14-days adaptation period is recommended for changeover and crossover experiments with cattle fed tropical forage-based diets.

RESUMO

MACHADO, Marcelo Grossi, M.Sc. Universidade Federal de Viçosa, dezembro de 2015. **Impactos da inversão de dietas sobre o consume voluntário, composição fecal e da digesta e perfil microbiano no rúmen de bovinos alimentados com dietas baseadas em forragens tropicais**. Orientador: Edenio Detmann. Coorientadores: Hilário Cuquetto Mantovani e Sebastião de Campos Valadares Filho.

Objetivou-se avaliar o impacto da inversão de dietas sobre o consumo voluntário, composição das fezes e digesta ruminal, fermentação ruminal e estrutura da microbiota ruminal de bovinos alimentados com dietas baseadas em forragens tropicais e estabelecer a duração mínima do período de adaptação que poderia ser aplicado em protocolos de experimentos conduzidos em delineamentos em crossover e changeover. Doze bovinos mestiços Europeu × Zebu fistulados no rúmen foram utilizados no experimento (quatro animais jovens não-castrados, quatro animais castrados e quatro novilhas). O experimento teve a duração total de 60 dias e duas inversões de dietas foram realizadas no 21º e 41º dias. Quatro sequências de dietas foram avaliadas: cana-se-açúcar-silagem de milho-cana-de-açúcar, silagem de milho-cana-de-açúcar-silagem de milho, baixo concentrado-alto concentrado-baixo concentrado e alto concentrado-baixo concentrado-alto concentrado. Todas as dietas foram ajustadas com ureia para apresentarem 110 g de proteína bruta por kg de matéria seca. Quatro grupos de variáveis foram avaliados ao longo do experimento: características do consumo voluntário, excreção e composição fecal, composição da digesta ruminal e perfil de fermentação ruminal. O comportamento dos grupos ao longo do tempo foi interpretado por um procedimento de agrupamento não-hierárquico. A diversidade das populações bacterianas da fase líquida da digesta ruminal foram avaliadas por PCR-DGGE somente nos animais jovens não castrados entre 6 dias antes e 20 dias após a segunda inversão de dietas. Variações na estrutura da comunidade bacteriana foram avaliadas por intermédio de procedimento de agrupamento utilizando-se o método de pares de grupos não-ponderados com média aritmética. A estabilização do consumo voluntário, digesta ruminal, excreção e composição fecal e fermentação ruminal ocorreu entre 9 e 13, 9 e 14, 6 e 13, e 4 e 11 dias após a inversão de dietas, respectivamente. A comunidade bacteriana na fase líquida estabilizou entre 3 e 9 dias após a inversão das dietas. O tempo máximo requerido para adaptação entre todas as características deve ser escolhido para assegurar-se a utilização de

protocolo experimental robusto. Assim, a partir dos resultados do presente trabalho, entre todos os tempos necessários para a adaptação do consumo, excreção fecal, composição e perfil de fermentação ruminal e microbiota ruminal, períodos de adaptação de 14 dias são recomendados para experimentos em changeover e crossover com bovinos alimentados com dietas baseadas em forragens tropicais.

Running head: Diet inversion in cattle

Impacts of diet inversion on voluntary intake, digesta and fecal composition, and bacterial community composition in rumen of cattle fed tropical forage-based diets[1]

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ABSTRACT: The objective of this study was to evaluate the impact of the diet inversion on voluntary intake, ruminal digesta and feces composition, ruminal fermentation, and bacterial community composition in cattle fed tropical forage-based diest and to establish the minimal length of the adaptation period that could be applied to changeover or crossover design protocols. Twelve rumen-cannulated crossbreed (B. taurus × B. indicus) bovines were used in this experiment (four young bulls, four steers, and four heifers). The experiment lasted 60 days and two diet inversions were done at d 21 and d 41. Four diet sequences were evaluated (one animal of each category per sequence): sugarcane-corn silage-sugarcane, corn silage-sugarcane-corn silage, high-low-high concentrate diets, and low-high-low concentrate diets. All diets were adjusted to 110 g CP/kg DM by using urea. Four group of variables were evaluated along experimental days: voluntary intake characteristics, fecal excretion and composition, ruminal digesta compostion, and ruminal fermentation profile. The pattern of the groups along time was interpreted through a non-hierarchical clustering procedure. The diversity of the liquid-associated rumen bacterial community was evaluated by PCR-DGGE in the young bulls from 6 days before to 20 days after the second diet inversion. The pattern of microbial diversity was evaluated through a clustering procedure using the unweight pair group method with arithmetic mean. Stabilization of the voluntary intake, ruminal digesta, fecal excretion and composition, and rumen fermentation profile was achieved within 9-13, 9-14, 6-13, and 4-11 days after diet inversion, respectively. The bacterial community in the liquid phase stabilized within 3-9 days after diet inversion. The maximum required adaptation period among all characteristics must be chosen to assure the utilization of a robust experimental protocol. From the results of this experiment, among all obtained times to adapt intake, fecal and digesta composition, and ruminal fermentation and microbial diversity, a 14-days adaptation period is recommended for changeover and crossover experiments with cattle fed tropical forage-based diets.

Key words: adaption period, changeover designs, carry over effect, DGGE

INTRODUCTION

Nutritional research with ruminant animals is known to be time-consuming, laborious and expensive. Changeover (e.g., Latin square) and crossover (e.g., switch-back) experiments are widely used in digestion trials and are important for decreasing the number of animals without compromising the number of experimental units. Nevertheless, one important object of attention must be the adaptation period within each experimental period of the experiment. This period is important to avoid or, at least, to control carry-over effects from previous treatments, which cause misunderstanding or confounding between the direct and residual effects of the treatments (Lucas, 1957; Grant et al., 2015).

Few studies have been done in the last decades toward defining the adequate length of the adaptation period for changeover or crossover designs. It has been found in the literature adaptation periods in ruminants digestive trials ranging from 7 to 27 d (Fonseca et al., 2006; Pérez-Ruchel et al., 2006; Archibeque and Huntington, 2015), being 14 d the modal value.

However, all aspects of the adaptation period are not completely clarified. Most recommendations for experimental protocols are based on specific groups of characteristics (e.g., Valadares Filho et al., 2000; Oliveira et al., 2001, DMI; Sutton et al., 2003, VFA; Fernando et al., 2010; Chen et al., 2011, microbial population) and an integrated view considering voluntary intake, chemical composition of samples, rumen fermentation and microbial characteristics simultaneously could be useful to define an adequate adaptation periods for digestion trials.

The objective of this study was to evaluate the impact of the diet inversion on voluntary intake, ruminal digesta and feces composition, ruminal fermentation and bacterial community composition in cattle fed tropical forage-based diet and to establish the minimal

length of the adaptation period that could be applied to changeover or crossover design protocols.

MATERIAL AND METHODS

This study was carried out at the Animal Science Department of the Universidade Federal de Viçosa, Brazil. All surgical and animal care procedures were conducted according to Committee of Ethics on Animal Handling guidelines of Universidade Federal de Viçosa (CEUAP, protocol no. 84/2014).

Animals and housing

Twelve crossbreed (Bos taurus × Bos indicus) bovines were used in this experiment: four young bulls averaging 241±19 kg BW and 1.5 year old, four steers averaging 431±26 kg BW and 2 years old, and four heifers averaging 288±11 kg BW and 1.8 year old. The animals were surgically fitted with ruminal canullae 30 ds prior the experiment and kept in individual stalls (2- × 5-m) with concrete floors and equipped with individual feeders and water dispensers. The animals had ad libitum access to fresh water and mineral mixture.

Experimental design and treatments

The trial was carried out according to a randomized block design with the blocks being the categories of the animals (heifers, steers and bulls), four treatments and three replicates per treatment.

The treatments consisted of four different sequences of diets: **SCS** (inversion of sugarcane to corn silage and back to sugar cane), **CSC** (inversion of corn silage to sugarcane and back to corn silage), **HLH** (inversion of a high-concentrate diet to a low-concentrate diet and back to high-concentrate diet), and **LHL** (inversion of a low-concentrate diet to a high-

concentrate diet and back to low-concentrate diet). The diets based on sugarcane and corn silage were composed only of the forages. The low- and high-concentrate diets were composed by corn silage with different forage to concentrate ratios (90:10 and 50:50 on a DM basis). Therefore these four sequences were based on two kinds of inversion: one between forages with different characteristics without concentrate, and another with inversion of different forage to concentrate ratios (Table 1). The CP protein content of the forages was corrected up to 110 g CP/kg DM using a mixture of urea:ammonium sulfate (9:1). The concentrate was based on corn and soybean meal to present also 110 g CP/kg DM. In this way, all diets were isonitrogenous (110 g CP/kg DM). Twice daily (0700 and 1600h) the animals were fed ad libitum with the respective diets allowing approximately 100 g/kg in orts.

Each diet was given for 20 d throughout a total experiment lasting 60 d. The diet inversions took place at d 21 and d 41.

Samples collection

The voluntary intake was measured during all 60 days of the experiment. The offered feeds and orts were weighed and sampled every day and frozen (-20ºC) for later chemical analyses.

The sampling of ruminal contents and feces began at d 16 (five days before the first diet inversion) and lasted until the d 60 (20 days after the second diet inversion).

Total fecal collection was performed for 45 days beginning at 600h. Feces were collected after spontaneous defecation and packed in polyethylene containers. After each 24 hours, the feces were homogenized and weighed. Representative samples (approximately 100 g/kg) were taken and frozen (-20ºC) for subsequent chemical analysis.

Sampling of ruminal contents were performed from d 16 to d 60 at 1200h. Samples were manually collected at several points of the liquid-solid interface of the rumen mat. A

200-g sample of ruminal content was homogenized and immediately frozen (-20ºC) for subsequent chemical analysis. A second sample of approximately 500 g was filtered through a triple layer of cheesecloth. The filtered liquid was split into three portions. The first portion of 20 mL was fixed with 1 mL of H_2SO_4 (50% v/v) and frozen for posterior analysis of ruminal ammonia nitrogen (**RAN**). The second portion of 8 mL was fixed with 2 mL of a meta-phosphoric acid solution (250 g/mL) and kept at -20ºC for subsequent VFA analysis. The third portion of 50 mL was transferred into polyethylene flask and frozen (-80ºC) for assessment of bacterial community composition.

Chemical analysis

The samples of feeds, orts, feces, and ruminal contents (for chemical analysis) were thawed at room temperature and oven-dried (55ºC). Samples were then processed in a knife mill (TE-650, Tecnal, Piracicaba, SP, Brazil) to pass through a 2-mm screen sieve. Approximately 50% of the ground material was processed again in the same mill to pass through a 1-mm screen sieve.

Chemical analyses were performed on the material processed to pass through a 1-mm sieve. The contents of DM (dried overnight at 105°C; method INCT-CA no. G-003/1), CP (Kjeldhal procedure; method INCT-CA no. N-001/1), ash (complete combustion in a muffle furnace at 600°C for 4 h; method INCT-CA no. M-001/1), NDF corrected for ash and protein (**NDFap**; using a heat-stable α-amylase, omitting sodium sulfite and correcting for residual ash and protein; method INCT-CA no. F-002/1), were quantified according to the standard analytical methods of the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA; Detmann et al., 2012; Table 1). The indigestible NDF (**iNDF**) contents were evaluated as the residual NDF remaining after 288 h of ruminal in situ incubation using F57 filter bags (Ankom Technology Corp., Macedon, NY), according to Valente et al. (2011).

The RAN concentration in rumen fluid was quantified using the colorimetric technique described by Chaney and Marbach (1962).

For VFA analysis (acetate, butyrate, and propionate), rumen fluid samples were centrifuged (12,000 × g, 10 min, 4°C), and supernatants were treated as described by Siegfried et al. (1984). Ruminal VFA were analyzed by HPLC by HPLC in a Dionex Ultimate 3000 Dual detector HPLC (Dionex Corporation, Sunnyvale, CA, USA) coupled to a refractive index (RI) Shodex RI-101 maintained at 40°C using a ion exchange column Phenomenex Rezex ROA, 300 × 7.8 mm maintained at 45°C. Mobile phase was prepared with 5 mmol/l sulfuric acid (H2SO4) and the flow was 0.7 ml/min. The following organic acids were used for the calibration of the standard curve: acetic (20 mmol/L), propionic (10 mmol/L), and butyric acid (10 mmol/L).

Bacterial community composition analysis

The four young bulls were chosen for this analysis because they were supposed to be more representative of cattle under production conditions. The samples used for these evaluations were taken around the second diet inversion in the experiment as follow: -3, -1, 0 (diet inversion day), +1, +3, +6, +9, +12, and +20 days after diet inversion.

For DNA extraction, the samples were defrosted at room temperature and processed according to the methodology described by Stevenson and Weimer (2007). Genomic DNA extracted from the liquid phase was utilized in the amplification reactions using primers specific for the regions V3-V4 and V4-V5 of the 16S rRNA of the Firmicutes and Bacteroidetes phyla, respectively (Muhling et al., 2008).

The PCR reactions were performed in a thermocycler Biocycler MG96G (São Paulo, Brazil) and the amplification reaction contained GoTaq Reaction Buffer (0.5 X), $MgCl_2$ (0.5 mmol/L), dNTPs (0.2 mmol/L), forward primer (0.12 mmol/L), reverse primer (0.12

mmol/L), Taq DNA polymerase (0.1 u/µL) (Promega Corporation, Madison, WI, USA), BSA (0.08 mg/mL) and genomic DNA (0.8 ng/µL). The PCR was performed with an initial temperature of denaturation at 96°C for 4 min followed by 35 cycles of 96°C for 1 min for denaturation, 56°C for 1 min for annealing of the primers and an additional 2 min at 72°C for primer extension. The amplification cycle was followed by a final extension step at 72°C for 5 min (Muhling et al., 2008). For the amplifications carried out with the primer for the Firmicutes and Bacteroidetes phyla, the annealing temperature was 50°C and 56°C, respectively.

To increase the specificity of the analysis, a nested-PCR was performed to amplify a shorter region of the Firmicutes and Bacteroidetes phyla (Muhling et al., 2008). The amplification reaction contained GoTaq Reaction Buffer (0.5 X), $MgCl_2$ (0.5 mmol/L), dNTPs (0.2 mmol/L), forward primer (0.12 mmol/L), reverse primer (0.12 mmol/L), Taq DNA polymerase (0.1 u/µL) (Promega Corporation, Madison, WI, USA) and BSA (0.08 mg/mL). One microliter of the amplification product from the first reaction was used as the DNA template. The nested-PCR was performed in the thermocycler Biocycler MG96G with an initial denaturation temperature of 96°C for 4 min, followed by 35 cycles at 96°C for 1 min for denaturation, 56°C for 1 min for annealing of the primers and 72°C for 30 s for primer extension. The amplification cycle was followed by a final extension at 72°C for 5 min (Muhling et al., 2008). For the amplifications carried out with the primer for the Firmicutes and Bacteroidetes phyla, the annealing temperature was 56°C and 52°C, respectively.

The denaturing gradient gel electrophoresis analysis (**DGGE**) was performed in a DGGE-2401 apparatus (CBS Scientific Company, San Diego, CA, USA) using 8 µL of the PCR products from the nested-PCR and 8 µL of sample buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol and 1X TAE (40 mmol/L Tris, 20 mmol/L acetic acid, and 1 mmol/LEDTA)). The PCR products were loaded into wells in a 8% (w/v) vertical

polyacrylamide gel (acrylamide:N,N'-methylenebisacrylamide, 37.5:1) with a linear gradient of 40 to 60% urea/formamide.

The denaturing gradient was obtained by mixing two solutions (A and B) dispensed by an MPP-100-220 peristaltic mini-pump (CBS Scientific Company, San Diego, CA, USA). Solution A contained 100% of the denaturing agents (7 mol/L urea and 40% deionized formamide (v/v)) in 8% acrylamide: N,N'-methylenebisacrylamide (37.5:1), and solution B was prepared as for solution A but without the denaturing agents. Solutions A and B also contained ammonium persulfate (3.1 mmol/L) of polymerizer and N,N,N',N'-tetramethylethylenediamine (3.7 µmol/L) catalyst. The denaturing gradient was monitored using 20 µL of the visualization dye (bromophenol blue 0.5%, xylene cyanol 0.5%, and 1X TAE). The gels were allowed to polymerize for 3 h prior to loading the DNA samples.

A mixture of 16S rRNA amplicons obtained from the genomic DNA of Escherichia coli ATCC 29214 (γ-proteobacteria), Salmonella enterica Typhimurium ATCC 14028 (γ-proteobacteria), Bacillus cereus ATCC 14579 (Firmicutes) and Lactococcus lactis ATCC 19435 (Firmicutes) were used as markers for bacterial species in wells located in the sides of the gel. Electrophoresis was performed at 60°C in 1 X TAE at constant voltage of 100 V for 15 h. The gel was stained for 20 min with SYBR Gold (Invitrogen, Breda, The Netherlands) according to manufacturer's recommendations. The gel was visualized and photo-documented using Eagle Eye (Stratagene, La Jolla, CA, USA). Gel bands were analyzed using Bionumerics 7.5 (Applied Maths, Kortrijk, Belgium).

Statistical analyses

The voluntary intake and ruminal and fecal characteristics were initially evaluated according to a randomized blocking design including the fixed effects of the diet sequences, days of sampling, and their interaction, and the random effect of blocks (animal categories)

using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Carry, NC, USA). The measures taken on different days were considered repeated measures over time. The choice of the best (co)variance structure was based on corrected Akaike's Information Criterion. Most of variables were analyzed using a heterogeneous compound symmetry matrix. It was adopted 0.05 as the critical level of probability for type I error.

It must be brought into evidence that most variables presented a significant interaction between sequence of diets and day of evaluation (Table 2, $P < 0.05$).

To interpret the interactions, the least square means for each variable within each sequence of diets were estimated using the MIXED procedure and standardized by using the STANDARD procedure of SAS 9.4 so that each group of least square means presented mean value 0 and standard deviation 1. The standardized groups of means were submitted to a multivariate clustering procedure according to the day of evaluation that was individually performed for each sequence of diets. It was chosen the non-hierarchical k-means clustering method (Johnson and Wichern, 1998; Katthree and Naik, 2000) by using the FASTCLUS procedure of SAS 9.4.

To perform the clustering procedures the response variables were previously organized in four different groups as follow:

1.Voluntary intake characteristics (**VIC**): OM intake (kg/d**)**, CP intake (kg/d), NDFap intake (kg/d), and iNDF intake (kg/d);

2.Fecal excretion and composition (**FEC**): fecal excretion (kg DM/d), fecal OM content (g/kg DM), fecal CP content (g/kg DM), fecal NDFap content (g/kg DM), and fecal iNDF content (g/kg DM);

3. Ruminal digesta compostion (**RDC**): ruminal digesta OM content (g/kg DM**)**, ruminal digesta CP content (g/kg DM), ruminal digesta NDFap content (g/kg DM), and ruminal digesta iNDF content (g/kg DM); and

4. Ruminal fermentation profile (**RFP**): total VFA concentration (mmol/L); molar proportion (mmol/100 mmol) of acetate, propionate and butyrate; acetate to propionate ratio; and RAN concentration (mg/dL).

Initially, a maximum of 10 clusters was established for each clustering procedure. If this choice became to create at least one cluster with only one observation (day), a new clustering was made with a maximum of 9 clusters. This procedure was repeated until it was found each and every cluster with at least two observations (days).

The efficiency of clustering was also evaluated through the over-all R^2 and the cubic clustering criterion (**CCC**). The contribution of each variable for discrimination among clusters was estimated by calculating the ratio between-cluster variance to within-cluster variance, which is proportional to the contribution for discrimination among clusters (Johnson and Wichern, 1998; Katthree and Naik, 2000).

To organize the cluster graphics, the numeric codes of the clusters were assigned following the numeric pattern of the variable that presented the highest ratio of between-cluster variance to within-cluster variance. From this, the cluster which presented the more negative deviation for that specific variable was assigned with the number 1 and so on.

The bacterial community composition was evaluated according to Pearson correlation coefficient and unweight pair group method with arithmetic mean (**UPGMA**) for cluster analysis (Popova et al., 2011). The time necessary to adapt bacterial community was identified from a clade that included the sample taken at d 20 after diet inversion.

RESULTS

There were interactions between diet sequences and days for all variables analyzed ($P \leq 0.01$), excepting for ruminal content of NDFap ($P = 0.52$) and VFA concentration ($P = 0.28$;

Table 2). Such a pattern demonstrates the necessity to take into account for the day-by-day variation for each kind of diet inversion separately.

In this way, all variables showed that they could be logically clustered within a non-hierarchical system that would facilitate the interpretation of the all 19 variables. All characteristics of non-hierarchical clustering are presented in Table 3.

The number of clusters for each diet sequences and group of variables varied from 4 to 6, being 6 the modal value (Table 3). The over-all R^2 ranged from 0.640 to 0.838. Considering the naturally high among-days variation for some responses, such as voluntary intake, the clustering presented a good fit to the different datasets. Such a pattern is reinforced by the high values of the CCC observed for all diet sequences and group of variables, which ranged from 2.028 to 15.458 (see Discussion).

For the first diet inversion using the HLH sequence (high-to-low concentrate), the VIC, FEC, RDC, and RFP achieved a stable pattern after 12, 14, 13, and 6 days, respectively (Fig. 1 and Table 4). For the second inversion (low-to-high concentrate), the FEC, RDC and RFP characteristics achieved a stable pattern after 12, 9, and 10 days, respectively. The VIC started a plateau after 9 days, but a small disturbance occurred in the d 16 to d 17 d after the second diet inversion, causing a movement of multivariate response to a close cluster which subsequently remained stable (Fig. 1). Accordingly, it is assumed the cluster changing that occurred at d 17 after second inversion would not correspond to a critical alteration on intake pattern and the stabilization point was assumed to occur at d 9 after second diet inversion.

For the first diet inversion using the LHL sequence (low-to-high concentrate), the stabilization for VIC, FEC, and RFP started at 10, 9, and 7 days after inversion, respectively. The RDC exhibited a stable pattern after 9 days, but a small disturbance occurred at d 17, before the response returned to the plateau at d 18. In this sense, it was assumed that stabilization did happen at d 9 d after inversion (Fig. 2, Table 4). For the second diet inversion

(high-to-low concentrate), VIC, FEC, RDC, and RFP achieved a stable pattern at 13, 9, 9, and 8 days after diet inversion.

Regarding CSC sequence, the stable patterns for VIC, FEC, and RPF were achieved at d 10, 9, and 4 after the first diet inversion, respectively (sugarcane-to-corn silage inversion; Fig. 3, Table 4). The RDC tended to become stable at d 8 d after inversion. In spite of this, after 8 days of stability, a small disturbance occurred at d 16 after inversion. The profile returned to the same cluster and remained stable for four more days and a small disturbance was observed again. However, it seems likely to assume that stable pattern started at d 8 after first diet inversion. For the second diet inversion (corn silage-to-sugarcane inversion), VIC, FEC, and RFP achieved stabilization after 11, 9, and 11 d after inversion. The RDC stabilized after 11 d. However, after five days exhibiting a stable pattern, RDC moved to a close cluster and then became stable. Nevertheless, it seems fair to state that stabilization started at d 11 after second diet inversion (Fig. 3).

For the SCS sequence, the VIC and FEC stabilized at d 10 d 6 after the first diet inversion, respectively (corn silage-to-sugarcane inversion; Fig. 4, Table 4). The RDC stabilized at d 6 after diet inversion and remained stable for 12 days, displaying only a small disturbance at d 18 after inversion, but immediately returned to the previous cluster. The RFP displayed a stable pattern at d 6 after inversion. Conversely, a disturbance happened after eight days of stabilization, and the multivariate response moved to a close cluster. Similarly to other responses obtained here, it is assumed this cluster change would not correspond to a critical alteration on ruminal fermentation profile and the stabilization point was assumed to occur at d 6 after first diet inversion. For the second diet inversion (sugarcane-to-corn silage inversion; Fig. 4), the VIC, FEC, RDC, and RFP stabilized at d 9, d 9, d 9, and d 11 after inversion.

Regarding microbial adaptation, the high-to-low concentrate diet inversion generated two distinct clades for Bacteroidetes phylum (Fig. 5a). Despite of the presence of the -1 day sample, the clade 2 grouped samples from +6 to +20 days after diet inversion, revealing greater microbial similarity with regards the Bacteriodetes phylum from +6 d after inversion. For the same diet inversion, the evaluation of Firmicutes phylum exhibited four main clades with clade 1 grouping the +9, +12, and +20 days after inversion, indicating that Firmicutes phylum populations stabilized from d 9 after diet inversion (Fig. 5b).

The Bacteroidetes phylum clustering for low-to-high concentrate inversion revealed three main clades (Fig. 6a). The clade 2 encompassed +6, +9, +12, and +20 days after inversion, showing microbial adaptation from d 6 after inversion. The Firmicutes phylum also displayed three main clades, but the adaptation seemed to occur after 9 days of diet inversion according to information from clade 3 (Fig. 6b).

To the sugarcane-to-corn silage inversion (Fig. 7a) the clade 2 encompassed +9, +12, and +20 days after inversion, revealing adaptation from d 9 after inversion. Regarding Firmicutes phylum one clade was composed by samples taken +6 to +20 days after inversion (Fig. 7b).

As regards the corn silage-to-sugarcane inversion displayed a more spread clustering pattern with regards Bacterioidetes phylum (Fig. 8a). Three main clades were grouped and a characteristic clade was formed by +9, +12, and +20 days after inversion, in despite of the presence of d 0. Concerning Firmicutes phylum, two main clades were observed and clade 2 was composed by samples taken from +3 days after inversion (Fig. 8b).

DISCUSSION

The presence of significant interactions between diet sequences and days for almost all variables associated with intake, digesta and feces composition, and rumen fermentation

would be expected, as the response to diet inversion seems to be dependent on the composition of each diet and on the nutritional discrepancy between previous and currently offered diets. Nonetheless, NDF concentration in rumen digesta and total VFA concentration in the rumen fluid did not present a significant interaction between diet sequences and days.

One of the possible causes for this lack of a significant interactions seems to be associated with high estimates of standard errors for these variables, which exhibited the lowest precision among all evaluated variables. On the other hand, in spite of some results obtained in non-tropical conditions reported significant alterations in total VFA concentration according to diet exchange (Allison et al., 1964; Tremere et al., 1968; Storry and Sutton, 1969), several studies conducted in the tropics have shown that different diets have limited impact on total VFA concentrations, but more prominent alterations has been observed on molar proportions of different acids (Figueiras et al., 2015; Franco, 2015; Batista et al., 2016), which agrees with the results obtained here. Such characteristics could be caused by the greater physical effectiveness and lower degradation of NDF from tropical forages.

On the other hand, neutral detergent soluble fraction (**NDS**) is readily degraded in the rumen, contrarily to NDF, which will be slower degraded and present a greater rumen retention time (Van Soest, 1994). In spite of different diets cause differences in the NDF pool size in the rumen (Huhtanen and Jaakkola, 1993), the faster disappearance of NDS makes the NDF concentration in the rumen digesta greater than in the diet and probably decreases the differences between diets with regard NDF concentration in ruminal digesta. In this way, it seems likely that NDF concentration in rumen digesta should not vary among diet sequences and days. In spite of this, there was a significant interaction regarding fecal NDF concentration. The amount of fiber leaving the rumen will vary according to the intake and ruminal digestibility for each diet. Therefore, even presenting similar rumen concentrations, the rumen outflow of fiber would not be similar for the different diets. Moreover, it must be

highlighted that any factor that decreases NDF degradation in the rumen will also increase the escape of undigested fiber from rumen and the fiber digestion in hindgut (Khalili and Huhtanen, 1991; Tesfa, 1993), which could also increase the variability of fiber concentration in feces among different diets. This seems be plausible here because diets varied regarding concentrate content and fiber quality.

The CCC is used to measure the success and accuracy of the clustering procedure. According to Katthree and Naik (2000), a CCC greater than 3 indicates a better clustering procedure compared to solutions when this value in lower than 3. In this work, it was achieved 13 of 16 clustering procedures with CCC greater than 3. Only three clusterings did not achieve that value, all of them in RDC group. This pattern may reflect a greater difficult to obtain homogenous samples of ruminal contents. In these specific cases, greater CCC could have been obtained by increasing the number of clusters. However, it would implicate obtaining a great number of clusters containing only one day, which would make more difficult the biological interpretation of data pattern. Therefore, one may infer that clustering procedures, in general, represented well the data.

In general, except for the first diet inversion for SCS diet sequence, the voluntary intake and ruminal digesta demanded similar times (averaging 10.5 and 9.3 days, maximum values 13 days for both) to stabilize after diet inversion. Such a pattern seems to be associated with the high influence of rumen fill on voluntary intake in cattle fed tropical forage-based diets (Detmann et al., 2014).

The carry-over effects on intake in changeover and crossover experimental designs are expected to be associated with short-term and intermediate-term aspects of voluntary intake control, as the experiments are not long enough to express long-term modifications in metabolic, physiological, and energy status of the animals. Short-term intake regulation is associated to meals within days and is noticeably parameterized by meal size and intermeal

intervals (Mertens, 1996; Allen, 2000), whereas intermediate-term regulation is characterized by variations among days, as the body reserves can act as buffers that accommodate intermediate and short-term deficits in intake (Mertens, 1996). Therefore, animals subjected to abrupt changes in diets will gradually respond to the new diet characteristics. Thus, time is necessary to complete the expression of the new intake pattern concerning a new diet.

The rumen is a mixing compartment (Ellis et al., 1994) where the resident mass is formed by residuals of different previous meals (Blaxter et al., 1956; Van Soest, 1994). In this context, the feeds of a meal from a new diet will mix with the resident mass and it will not be completely significant with regard fill effect as the previous meals would be still quantitatively and qualitatively influencing the kinetics of rumen outflow. The disappearance of undigested and indigestible residues tends to obey the first-order law that output is proportional to residual concentration, and so it declines asymptotically (Van Soest, 1994). Therefore, the new diet will exert predominant effect on rumen fill when its residues become a significant part of the rumen digesta, which, obviously, will depend on disappearance of the residuals from the previous diet. This is of particularly relevance to the tropics because tropical forages present higher fill effect compared to non-tropical forages. Moreover, it must be emphasized that diets used here were quite heterogeneous concerning NDF and iNDF contents. Thus, intake adaptation of a new diet is associated to ruminal digesta adaptation.

It should be expected that differences with regard rumen fill effect are more prominent concerning intermediate-term intake regulation (among days). However, results obtained with animals fed tropical forage-based diets showed that both physical and metabolic regulation act simultaneously on intake regulation (Detmann et al., 2014). In this sense, some metabolic short-term mechanisms can also be responsible for changes in intake pattern after a diet inversion and this will also take time to adapt the animal to the new diet. Alterations in the fermentability of substrates in the rumen can cause modifications in the availability of

metabolic fuels (e.g., propionate), which alters the feeding pattern (i.e., meal size, intermeal interval) and, consequently, voluntary intake (Oba and Allen, 2003a; 2003b; Grant et al., 2015). It is particularly relevant to inversion of diets with different forage-to-concentrate ratios. The average values of molar proportion of propionate (24.0 vs. 15.1 mmol/100 mmol) and the acetate-to-propionate ratio (2.87 vs 5.22) in high and low-concentrate diets were very discrepant in this study. It highlights that voluntary intake adaptation also depends on ruminal fermentation adaptation.

According to Grant et al. (2015), response to diet for eating, ruminating, and resting behaviors usually stabilizes within 1 to 7 days. These authors stated that voluntary intake response to diets reflects the pattern of eating behavior, but the time course is affected by physical and chemical attributes of the diet such as particle size, carbohydrate fermentability, and fat or protein content. They concluded that adaptation periods of approximately 7 to 14 days are usually adequate for measuring response in voluntary intake. This conclusion generally agrees with this work, as 9 to 13 days were required to stabilize voluntary intake characteristics.

On average, fecal excretion and composition stabilized approximately 0.7 day (16.8 hours) after stabilization of ruminal digesta composition. It is logical to affirm that fecal mass and feces composition will be influence by amount and composition of digesta leaving the rumen. The NDS will present a high true digestibility in the small intestine and the amount of fiber digested in the hindgut will be variable. The average lag between stabilization of rumen digesta and feces composition would be caused by the time of non-mixing flow through small intestine (Ellis et al., 1994) and by the mixing flow through caecum (Cruickshank et al., 1989). Results obtained with cattle fed tropical forage-based diets pointed out post-ruminal retention times ranging from 12 to 24 hours (Oliveira et al., 1999; Bürger et al., 2000; Pereira et al., 2003), which supports the results obtained here.

The evaluation of bacterial community composition in this study focused on Firmicutes and Bacterioidetes phyla because it is expected that they represent around 93% of the operational taxonomic units in the rumen (Jami and Mizrahi, 2012).

The change in ruminal microbial populations in response to a changed ration is an important factor influencing tolerance and adaptation to a new diet (Allison et al., 1964). In spite of the effects of the host on the structure of microbial populations in the rumen (Allison et al, 1964; Weimer et al., 2010; Jami and Mizrahi, 2012), it seems fair to assume that diet is the most impacting factor on microbial diversity in the rumen (Tajima et al., 2001; Menezes et al., 2011; Reis et al., 2015).

Dietary shifts disturb microbiota and fermentation of the rumen, and varying amounts of time are required for them to re-stabilize (Hackman, 2015). However, the descriptions of the necessary time to complete the adaptation of the ruminal environment to a diet change are quite variable (Annison et al., 1959, 3-6 days; Storry and Sutton, 1969, 7 days; Kaufmann et al., 1980, 10-14 days; Brown et al., 2006, 2-7 days). Nevertheless, most of the studies were based on few characteristics of rumen environment (e.g., molar proportion of VFA) and did not take into account for modifications in rumen fermentation pattern and changes in rumen microbiota simultaneously. In this study, rumen fermentation pattern and bacterial community composition stabilized within 4-11 days (averaging 7.9 days) and 3-9 days (averaging 7.2 days), respectively.

However, considering that microbial assessment was conducted only during the second diet inversion, it is noted that, for this inversion, adaptation of ruminal fermentation pattern lasted longer (averaging 10 days) than the adaptation of microbiota. According to Hackman (2015), stabilization of fermentation does not necessarily coincide with stabilization of the microbiota, which agrees with the results obtained here. This difference could be attributed to some adaptation of metabolic pathways or catabolic regulations of the

microorganisms to the changing of available substrates (Russell and Baldwin, 1978). On the other hand, some changes can be still occur in rumen microbiota with regard to class, genera, and bacterial species after stabilization of phyla pattern but before the stabilization of fermentation pattern. Such an alterations cannot be detected here as the evaluations were performed at phyla level.

It should also be noted that the stabilization of Firmicutes occurred later than Bacterioidetes when inversion was based on concentrate levels (9 vs. 6 d) and the opposite was observed for the inversion based on forage type (3-6 vs. 9 d). This pattern agrees with Hackman (2015), who stated that specific groups of microorganisms also require varying amounts of time to stabilize.

On the other hand, it must be highlighted the microbial evaluation performed here was based on liquid-associated bacteria. Most bacterial populations are associated with solid phase of rumen digesta (Henderson et al., 2013). It seems likely to affirm that microbial communities associated to the solids would show more stability over time and a longer adaptation to a diet change could be expected. Similarly, the methanogen community would depend on the adaptation of bacterial species that provide H_2 for its metabolism and also should display a longer adaptation to a diet change. Therefore, influence of diet inversion on solid-associated bacteria and methanogen communities still remain to be investigated in cattle fed tropical forages-based diets.

According to Harvatine (2015), information with regards time required for diet adaptation and minimization of carry-over effects in ruminants is invaluable to efficient experimental design. The results obtained here indicated that different characteristics can demand different adaptation times to be correctly measured after a diet change. Some influence of the difference between the previous and current diet offered to the animal can affect the adaptation time as well. In this sense, it should be understood that a robust protocol

must be defined as all characteristics can be measured after a complete stabilization with a concurrent minimization of carry-over effects. Therefore, the maximum required adaptation period among all characteristics to be measured must be chosen to assure that condition. From the results of this experiment, among all obtained times to adapt intake, fecal and digesta composition, and ruminal fermentation and bacterial community composition, a 14-d adaptation period is recommended for changeover and crossover experiments with cattle fed tropical forage-based diets.

REFERENCES

Allison, M. J., J. A. Bucklin, and R. W. Dougherty. 1964. Ruminal changes after overfeeding with wheat and the effect of intraruminal inoculation and adaptations to a ration containing wheat. J. Anim. Sci. 4:1164-1171. doi:10.2134/jas1964.2341164x

Annison, E. F., D. Lewis, and D. B. Lindsay. 1959. The metabolic changes which occur in sheep transferred to lush spring grass. I. Changes in blood and rumen constituents. J. Agr. Sci. 53:34-41. 53:34-41. doi:.10.1017/S0021859600030896

Allen, M. S. 2000. Effects of diet on short-term regulation of feed intake by lactation dairy cattle. J. Dairy Sci. 83:1598-1624. doi: 10.3168/jds.S0022-0302(00)75030-2

Archibeque, S. L., and G. B. Huntington. 2015. The perspectives of a beef cattle nutritionist. . J. Anim. Sci. 98:313 (Suppl. s3).

Batista, E. D., E. Detmann, E. C. Titgemeyer, S. C. Valadares, R. F. D. Valadares, L. L. Prates, L. N. Rennó, and M. F. Paulino. 2016. Effects of varying ruminally undegradable protein supplementation on forage digestion, nitrogen metabolism, and urea kinetics in Nellore cattle fed low-quality tropical forage. J. Anim. Sci. (in press) doi: 10.2527/jas2015-9493

Blaxter, K. L., N. M. Graham, and F. W. Wainman. 1956. Some observations on the digestibility of food by sheep and on related problems. Brit. J. Nutr. 10:69-91.

Bürger, P. J., J. C. Pereira, J. F. C. Silva, S. C. Valadares Filho, P. R. Cecon, C. P. Jordão, and S. P. Braz. 2000. Passage rates and ruminal degradation kinetics in holstein calves fed diets with different concentrate levels. Rev. Bras. Zootecn. 29:225-235. doi: 10.1590/S1516-35982000000100030.

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8:103-132.

Chen, Y., G. B. Penner, M. Li, M. Oba, and L. L. Guan. 2011. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. App. Environ. Microbiol. 16:5770-5781. doi: 10.1128/AEM.00375-11

Cruickshank, G. J., D. P. Poppi, and A. R. Sykes. 1989. Theoretical considerations in the estimation of rumen fractional outflow rate from various sampling sites in the digestive tract. Brit. J. Nutr., 62:229-239.

Detmann, E., M. P. Gionbelli, and P. Huhtanen. 2014. A meta-analytical evaluation of the regulation of voluntary intake in cattle fed tropical forage-based diets. J. Anim. Sci. 92:4632-4641. doi: 10.2527/jas.2014-7717

Detmann, E., M. A. Souza, S. C. Valadares Filho, A. C. Queiroz, T. T. Berchielli, E. O. S. Saliba, L. S. Cabral, D. S. Pina, M. M. Ladeira, and J. A. G. Azevedo. 2012. Métodos para análise de alimentos. Suprema, Visconde do Rio Branco, Minas Gerais, Brazil.

Ellis, W. C.; J. H. Matis, T. M. Hill, and M. R. Murphy. 1994. Methodology for estimating digestion and passage kinetics of forages. In: Fahey Jr., G. C., editor, Forage quality, evaluation, and utilization. American Society of Agronomy, Madison, WI. p.682-756.

Fernando, S.C.; H. T. Purvis II, F. Z. Najar, L. O. Sukharnikov, C. R. Krehbiel, T. G. Nagaraja, B. A. Roe, and U. Desilva. 2010. Rumen microbial population dynamics during

adaptation to high-grain diet. Appl. Environ. Microbiol. 22:7482-7490. doi: 10.1128/AEM.00388-10

Figueiras, J. F., E. Detmann, S. C. Valadares Filho, M. F. Paulino, E. D. Batista, L. M. A. Rufino, T. N. P. Valente, W. L. S. Reis, and M. O. 2015. Nutritional performance of grazing cattle during dry-to-rainy transition season with protein supplementation. Arch. Zootec. 64:269-276.

Fonseca, C. E. M., R. F. D. Valadares, S. C. Valadares Filho, M. T. Rodrigues, M . I. Marcondes, M. O. Porto, D. S. Pina, and K. A. K. Moraes. 2006. Production of goats fed diets with increasing levels of protein: intake, milk, yield and apparent digestibility. Rev. Bras. Zootecn. 3:1162-1168. doi: 10.1590/S1516-35982006000400030

Franco, M. O. 2015. Nutritional performance and metabolic characteristics in cattle fed tropical forage and supplemented with nitrogenous compounds and energy. PhD Diss., Univ. Federal de Viçosa, Viçosa, Minas Gerais, Brazil

Grant R. J., H. M. Dann, and M. E. Woolpert. 2015. Time required for adaptation of behavior, feed intake and dietary digestibility in cattle. J. Anim. Sci. 93:312 (Suppl. s3).

Hackmann, T. 2015. Time required for adaptation of rumen fermentation and the rumen microbiome. J. Anim. Sci. 98:312 (Suppl. s3).

Harvatine, K. J. 2015. Time required for diet adaptation and minimization of carry-over effect in ruminants: The perspective of an experimental researcher. J. Anim. Sci. 98:312-313 (Suppl. s3).

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

Huhtanen P., and S. Jaakkola. 1993. The effects of forage preservation method and proportion

of concentrate on digestion of cell wall carbohydrates and rumen digesta pool size in cattle. Grass Forage Sci. 48:155-165. doi: 10.1111/j.1365-2494.1993.tb01848.x

Johnson, R. A. and D. W. Wichem. 1998. Applied multivariate statistical analysis. Prentice-Hall International, New Jersey, NY.

Jami, E., and I. Mizrahi. 2012. Composition and similarity of bovine rumen microbiota across individual animals. Plos One. 3:e33306. doi:10.1371/journal.pone.0033306

Katthree, R., and D. N. Naik. 2000. Multivariate data reduction and discrimination. SAS Institute Inc., Cary, NC.

Kaufmann W., H. Hagemeister, and G. Dirksen. 1980. Adaptation to changes in dietary compostition, level, and frequency of feeding. Pages. 586-592 in Digestive Physiology and Metabolism of Ruminants. Y. Ruckebusch and P. Thievend, editors. AVI Publishing Co., Inc., Westport, CT.

Khalili, H., and P. Huhtanen. 1991. Sucrose supplements in cattle giving grass silage-based diet. 1. Digestion of organic matter and protein. Anim. Feed Sci. Techol. 33:247-261. doi:10.1016/0377-8401(91)90064-Y

Lucas, H. L. 1957. Extra-period latin squares change-over designs. J. Dairy Sci., 3:225-239. doi: 10.3168/jds.S0022-0302(57)94469-7

Menezes, A. B., E. Lewis, M. O'Donovan, B. O'Neil, N. Clipson, and E. M. Doyle. 2011. Microbiome analysis of dairy cows fed pasture or total mixed ration diets. FEMS Microbiol. Ecol. 78:256-265. doi: 10.1111/j.1574-6941.2011.01151.x

Mertens, D. 1996. Methods in modeling feeding behavior and intake in herbivores. Ann. Zootech. 45:153-164. z

Muhling, M., J. Woolven-Allen, J. C. Murrell, and I. Joint. 2008. Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. The ISME Journal, 2:379–392. doi: 10.1038/ismej.2007.97

Oliveira, R. L., J. C. Pereira, P. R. C. Silva, and R. A. M. V. Vieira. 1999. Coast-cross hay and broiler litter ruminal degradation and evaluation of mathematical models to estimate the rate of particulate passage. Rev. Bras. Zootecn. 28:839-849. doi: 10.1590/S1516-35981999000400026

Oba, M., M. S. Allen. 2003a. Dose-response effects of intrauminal infusion of propionate on feeding behavior of lactating cows in early or midlactation. J. Dairy Sci. 86:2922-2931. doi: 10.3168/jds.S0022-0302(03)73889-2

Oba, M., and M. S. Allen, M. S. 2003b. Intraruminal infusion of propionate alters feeding behavior and decreases energy intake of lactating dairy cows. J. Nutr., 133:1094-1099.

Pereira, E. S., A. C. Queiroz, M. F. Paulino, S. C. V. Valadares Filho, A. M. V. Arruda, L. S. Cabral, L.F. Miranda, and A. M. Fernandes. 2003. Dynamic of the nutrients in the gastrintestinal tract of holstein steers fed sugarcane-based diets. Rev. Bras. Zootecn. 32:1524-2003. doi: 10.1590/S1516-35982003000600028

Pérez-Ruchel, A., J. L. Repetto, M. Michelini, L. Pérez, G. Soldini, and C. Cajarville. 2006. Rate of intake in wethers fed a temperature pasture with different feeding schedules and supplemented or not with additives. South African J. of Anim. Sci.. 5:157-160. doi: 10.4314/sajas.v39i1.61256

Popova M., C. Martin, M. Eugène, M. M. Mialon, M. Doreau, and D. P. Morgavi. 2011. Effect of fibre- and starch-rich finishing diets on methanogenic Archaea diversity and activity in the rumen of feedlot bulls. Anim. Feed Sci. Technol. 167:113-121. doi: 10.1016/j.anifeedsci.2011.04.060

Reis, W. L. S., E. Detmann, E. D. Batista, L. M. A. Rufino, D. I. Santos, C. B. P. Bento, H. C. Mantovani, and S. C. Valadares Filho. 2015. 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. Anim. Feed Sci. Technol. (submitted).

Oliveira, A. S., R. F. D. Valadares Filho, S. C. Valadares Filho, P. R. Cecon, G. A. Oliveira, R. M. N. Silva, and M. A. L. Costa. 2001. Intake, apparent digestibility, milk composition and production of lactating cows fed four non protein nitrogen compounds levels. Rev. Bras. Zootecn., 30:1358-1366. doi: 10.1590/S1516-35982001000500032

Russell, J. B., and R. L. Baldwin. 1978. Substrate preferences in rumen bacteria: evidence of catabolic regulatory mechanisms. Appl. Environ. Microbiol. 36:319-329.

Siegfried, B. R. H. Ruckermann, and G. Stumpf. 1984. Method for determination of organic acids in silage by high performance liquid chromatography. Landwirtsch. Forsch. 37:298-304.

Stevenson D. M., and P. J. Weimer. 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. and Biotech., 75:165-174. doi: 10.1007/s00253-006-0802-y

Storry, J.E., and J. D. Sutton. 1969. The effect of change from low-roughage to high-roughage diets on rumen fermentation, blood composition and milk fat secretion in the cow. Brit. J. Nut., 23:511-521.

Sutton, J. D., M. S. Dhanoa, S. V. Morant, J. France, D. J. Napper, and E. Schuller. 2003. Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. J. of Dairy Sci. 86:3620-3633. doi: 10.3168/jds.S0022-0302(03)73968-X

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population on the rumen revealed with real-time PCR. Appl. Environ. Microb. 67:2766-2774. doi: 10.1128/AEM.67.6.2766–2774.2001

Tesfa, A.T. 1993. Effects of rape-seed oil supplementation on digestion, microbial protein synthesis and duodenal microbial amino acid composition in ruminants. Anim. Feed Sci. Tecnhol. 41:313-328. doi: 10.1016/0377-8401(93)90005-5

Tremere, A. W., W. G. Merrill, and J. K. Loosli. 1968. Adaptation to high concentrate feeding as related to acidosis and digestive disturbances in dairy heifers. J. Dairy Sci. 51:1065-1072. doi: 10.3168/jds

Valadares Filho, S. C., G. A. Broderick, R. F. D. Valadares, M. K. Clayton. 2000. Effect of replacing alfalfa silage with high moisture corn on nutrient utilization and milk production. J. Dairy Sci. 83:106-114. doi: 10.3168/jds.S0022-0302(00)74861-2

Valente, T. N. P., E. Detmann, A. C. Queiroz, S. C. Valdares Filho, D. I. Gomes, and J. F. Figueiras. 2011. Evaluation of ruminal degradation profiles of forages using bags made from different textiles. Rev. Bras. Zootecn. 40:2565–2573. doi: 10.1590/S1516-35982011001100039

Van Soest, P.J. 1994. Nutritional Ecology of the Ruminant. 2 ed.Ithaca: Cornell University Press.

Weimer, P. J., D. M. Stevenson, H. C. Mantovani, and S. L. C. Man. 2010. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. J. Dairy Sci. 93:5902-5912. doi: 10.3168/jds.2010-3500

Table 1. Feed and chemical composition of the experimental diets

Item	Diets			
	Corn silage	Sugarcane	High concentrate	Low concentrate
Ingredient				
Corn silage, g/kg DM	1000	0	500	900
Sugarcane, g/kg DM	0	1000	0	0
Concentrate[1], g/kg DM	0	0	500	100
Chemical composition				
DM, g/kg as fed	239.6	286.9	370.9	256.7
OM, g/kg DM	923.6	977.1	945.4	927.7
CP, g/kg DM	108.0	105.9	108.0	108.1
NDFap[2], g/kg DM	537.3	445.6	339.3	501.0
iNDF[3], g/kg DM	243.6	304.6	146.1	227.7

[1] Concentrate composition (DM basis): 928.1 g/kg of corn, 52.5 g/kg of soybean meal and 19.4 g/kg of mineral mixture. [2] NDFap, NDF corrected for residual ash and protein. [3] iNDF, indigestible NDF.

Table 2. Descriptive levels of probability for type I error associated to the effects of sequences of diets, days of evaluation and their interaction

Item	Sequences of diets (S)	Days (D)	S × D	SEM
Voluntary intake				
OM, kg/d	<0.01	<0.001	<0.001	0.746
CP, kg/d	<0.01	<0.001	<0.001	0.089
NDFap[1], kg/d	<0.01	<0.001	<0.001	0.346
iNDF[2], kg/d	0.25	<0.001	<0.001	0.172
Fecal excretion and composition				
FE[3], kg DM/d	<0.001	<0.001	<0.001	0.303
OM, g/kg DM	<0.001	<0.001	<0.001	19.2
CP, g/kg DM	<0.001	<0.001	<0.001	5.7
NDFap[1], g/kg DM	<0.001	<0.001	<0.001	23.1
iNDF[2], g/kg DM	<0.001	0.03	<0.001	28.2
Ruminal digesta composition				
OM, g/kg DM	<0.001	<0.001	<0.001	6.0
CP, g/kg DM	<0.001	<0.001	<0.001	5.0
NDFap[1], g/kg DM	0.47	0.56	0.52	159.5
iNDF[2], g/kg DM	<0.001	<0.001	<0.001	22.9
Ruminal fermentation				
VFA, mmol/L	<0.01	<0.001	0.28	10.06
AC[4], mmol/100 mmol	<0.001	<0.001	<0.001	2.05
PROP[5], mmol/100 mmol	<0.001	0.007	<0.001	1.81
BUT[6], mmol/100 mmol	<0.001	<0.001	0.01	1.19
A:P[7]	<0.001	<0.001	<0.001	0.45
RAN[8], mg/dL	<0.001	<0.001	<0.001	2.78

[1]NDFap, NDF corrected for ashes and protein. [2] iNDF, indigestible NDF.[3]FE, fecal excretion. [4]AC, acetate. [5]PROP, propionate. [6]BUT, butyrate. [7]A:P, acetate to propionate ratio. [8]RAN, ruminal ammonia nitrogen.

Table 3. Characteristics of the clustering procedure according to the days of evaluation for the different sequences of diets

	Sequences of diets[1]			
Item	HLH	LHL	CSC	SCS
Voluntary intake[2]				
OM, kg/d	14.288	5.633	6.947	1.742
CP, kg/d	4.745	10.136	2.506	6.938
NDFap[3], kg/d	3.220	4.069	5.049	2.046
iNDF[4], kg/d	4.838	3.147	6.092	3.136
Number of clusters	6	6	5	6
Over-all R^2	0.838	0.830	0.820	0.735
CCC[5]	13.645	12.791	15.458	4.738
Fecal excretion and composition[2]				
FE[1], kg DM/d	1.657	2.373	1.731	1.131
OM, g/kg DM	5.420	0.818	5.631	3.344
CP, g/kg DM	2.463	2.389	2.582	1.194
NDFap[3], g/kg DM	6.229	1.010	2.674	5.720
iNDF[4], g/kg DM	5.128	5.208	6.450	5.121
Number of clusters	6	4	6	6
Over-all R^2	0.775	0.640	0.759	0.706
CCC[5]	10.382	9.505	9.067	13.970
Ruminal digesta composition[2]				
OM, g/kg DM	1.423	2.515	1,773	3.897
CP, g/kg DM	0.895	2.147	1547	3.926
NDFap[3], g/kg DM	1.608	0.738	1.688	2.555
iNDF[4], g/kg DM	1.216	6.602	4.190	3.304
Number of clusters	5	6	5	5
Over-all R^2	0.666	0.672	0.670	0.770
CCC[5]	2.905	2.213	2.028	7.965
Ruminal fermentation profile[2]				
VFA, mmol/L	1.525	3.198	1.505	1.007
AC[6], mmol/100 mmol	6.288	4.064	6.470	6.788
PROP[7], mmol/100 mmol	5.270	5.902	4.654	6.678
BUT[8], mmol/100 mmol	1.814	1.410	0.783	1.024
A:P[9]	4.731	6.311	2.709	7.142
RAN[10], mg/dL	1.067	1.143	5.354	1.794
Number of clusters	6	6	6	6
Over-all R^2	0.716	0.734	0.717	0.711
CCC[5]	10.284	11.737	10.393	9.958

[1]HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugar cane-corn silage-sugar cane; SCS, sequence corn silage-sugar cane-corn silage. [2]The values indicated for each variable within each sequence of diets correspond to the ratio of between-cluster variance to within-cluster variance, $R^2/(1-R^2)$. [3]NDFap, NDF corrected for ashes and protein. [4]iNDF, indigestible NDF. [5]CCC, cubic clustering criterion. [6]AC, acetate. [7]PROP, propionate. [8]BUT, butyrate. [9]A:P, acetate to propionate ratio. [10]RAN, ruminal ammonia nitrogen.

Table 4. Summary of the number of days necessary to stabilize the different characteristics associated with voluntary intake, fecal excretion, and ruminal fermentation and composition after diet inversion

Sequence of diets	Characteristics			
	Voluntary intake	Fecal excretion and composition	Ruminal digesta compostion	Ruminal fermentation profile
	1st diet inversion			
HLH	12	14	13	6
LHL	10	9	9	7
CSC	10	9	8	4
SCS	10	9	6	6
	2nd diet inversion			
HLH	9	12	9	10
LHL	13	9	9	8
CSC	11	9	11	11
SCS	9	9	9	11
Mean	10.5	10.0	9.3	7.9
SEM	0.50	0.68	0.73	0.91
Maximum	13	14	13	11

FIGURE CAPTIONS

Figure 1. Clustering results for sequence high concentrate-low concentrate-high concentrate diets (a, voluntary intake; b, fecal excretion and composition; c, ruminal digesta composition; d, ruminal fermentation profile). Diet changes occurred on 21st and 41st days.

Figure 2. Clustering results for sequence low concentrate-high concentrate-low concentrate diets (a, voluntary intake; b, fecal excretion and composition; c, ruminal digesta composition; d, ruminal fermentation profile). Diet changes occurred on 21st and 41st days.

Figure 3. Clustering results for sequence sugar cane-corn silage-sugar cane diets (a, voluntary intake; b, fecal excretion and composition; c, ruminal digesta composition; d, ruminal fermentation profile). Diet changes occurred on 21st and 41st days.

Figure 4. Clustering results for sequence corn silage-sugar cane-corn silage diets (a, voluntary intake; b, fecal excretion and composition; c, ruminal digesta composition; d, ruminal fermentation profile). Diet changes occurred on 21st and 41st days.

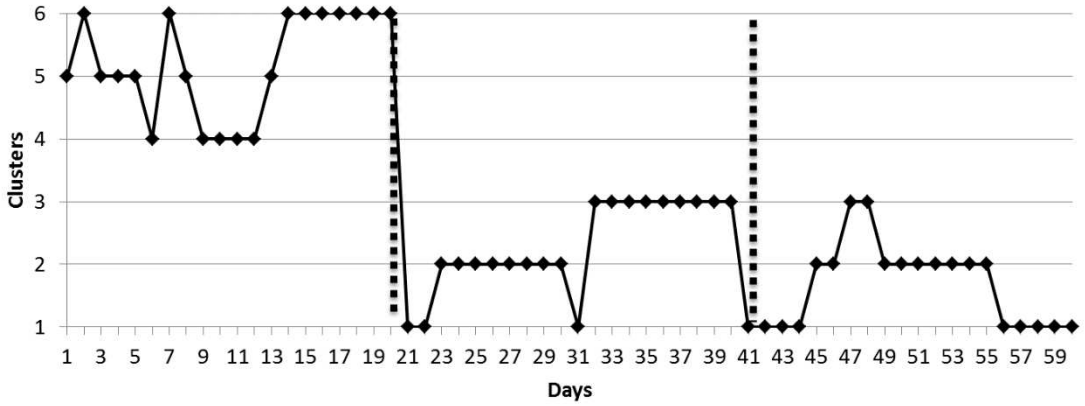
Figure 5. Clustering based on bacterial community composition obtained from DGGE analysis for high-to-low concentrate diets inversion (a, Bacterioidetes; b, Firmicutes)

Figure 6. Clustering based on bacterial community composition obtained from DGGE analysis for low-to-high concentrate diets inversion (a, Bacterioidetes; b, Firmicutes)

Figure 7. Clustering based on bacterial community composition obtained from DGGE analysis for sugarcane-to-corn silage diets inversion (a, Bacterioidetes; b, Firmicutes)

Figure 8. Clustering based on bacterial community composition obtained from DGGE analysis for corn silage-to-sugarcane diets inversion (a, Bacterioidetes; b, Firmicutes)

Figure 1



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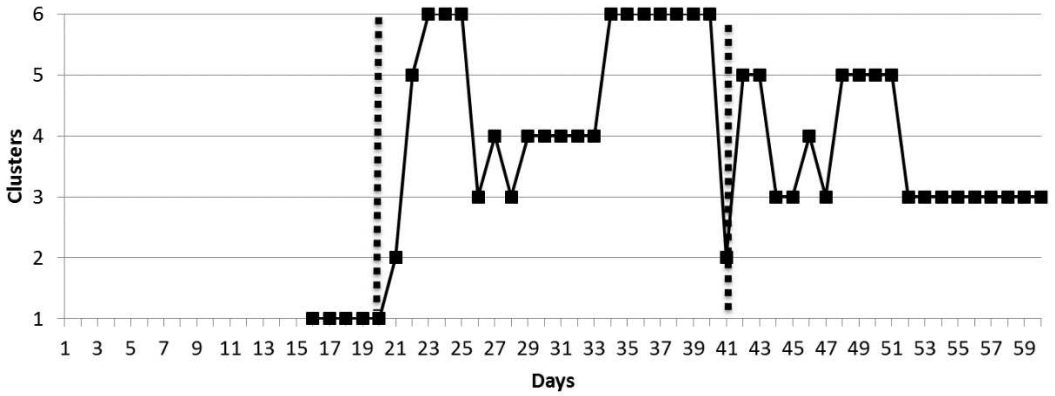

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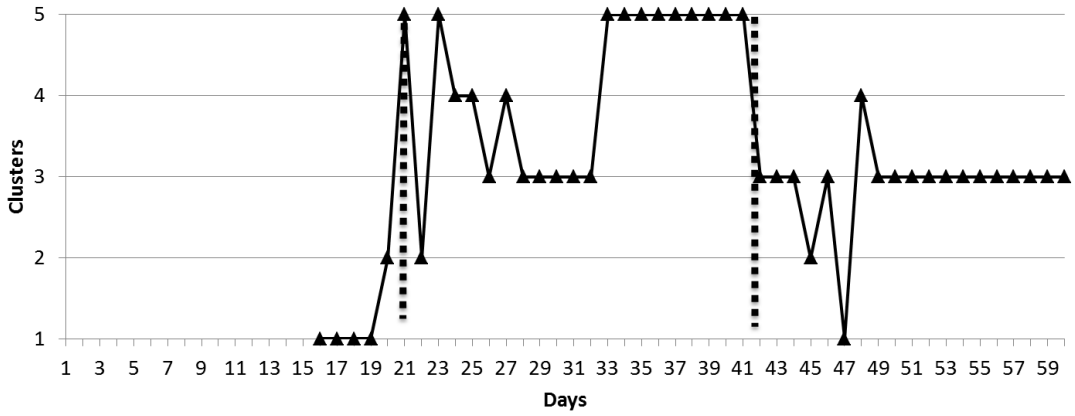

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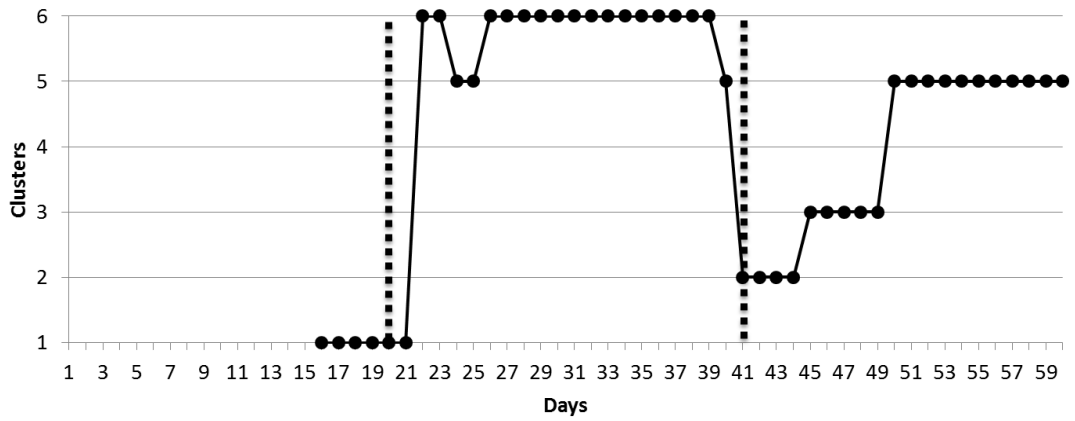

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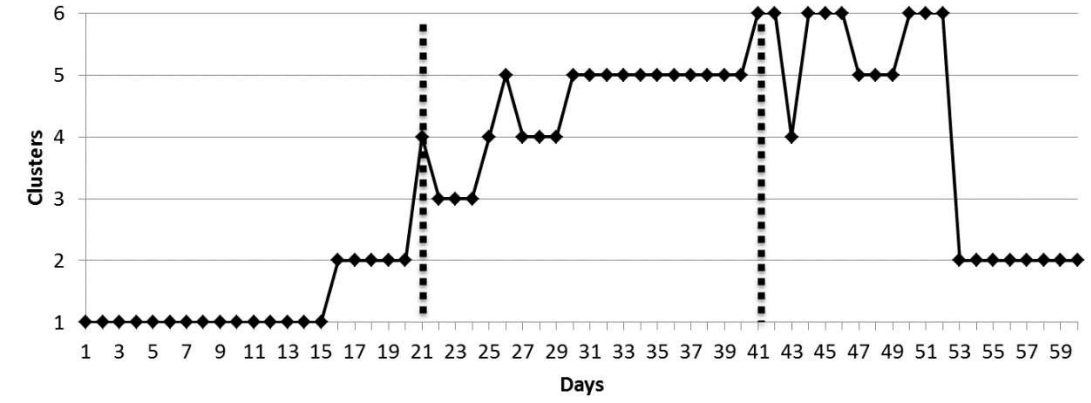

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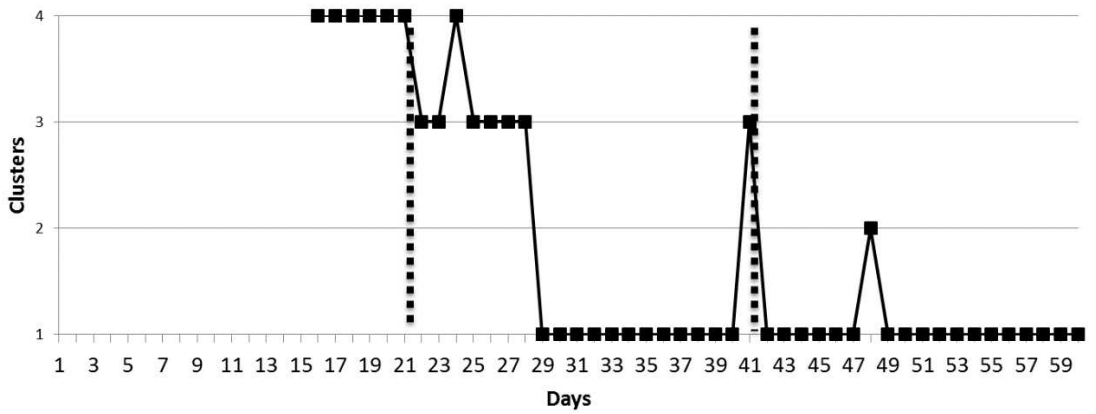

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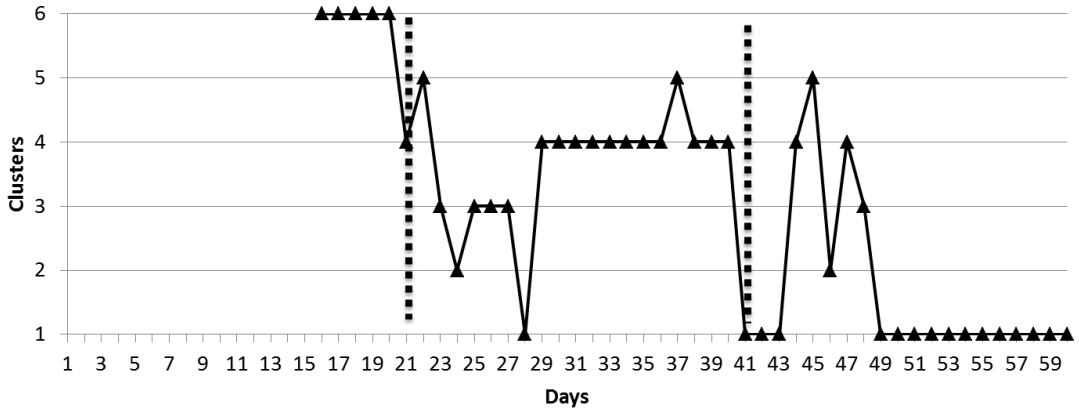

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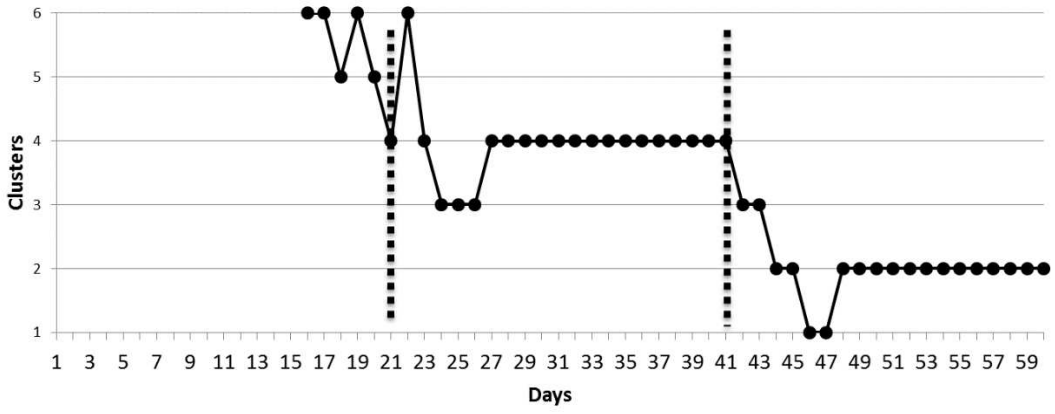

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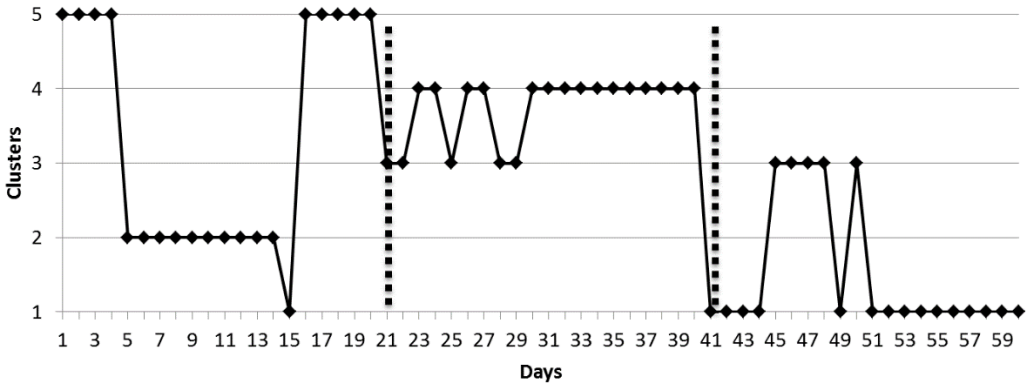

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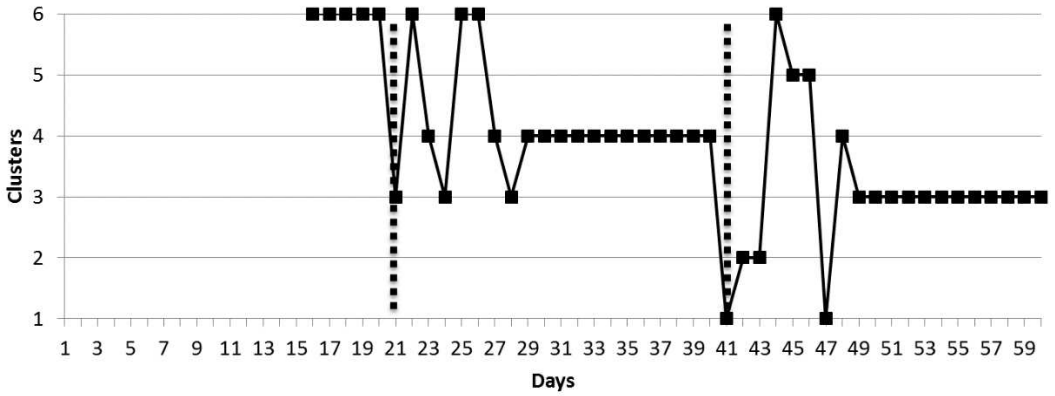

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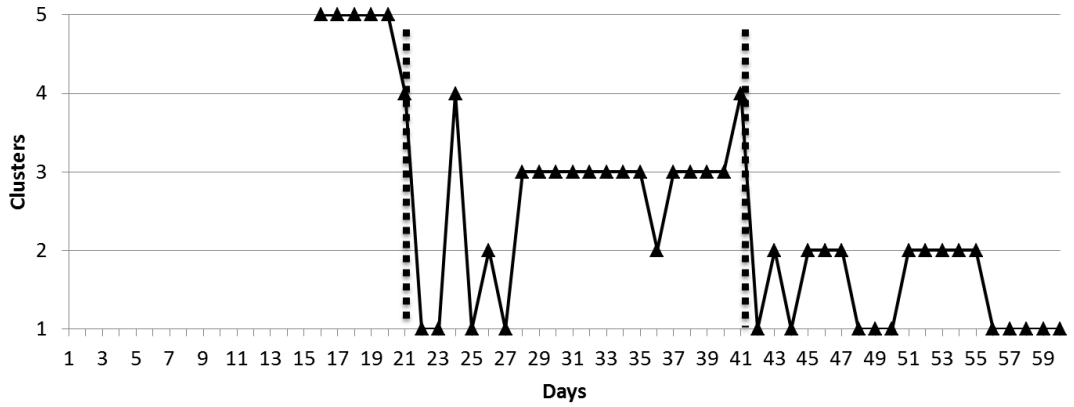

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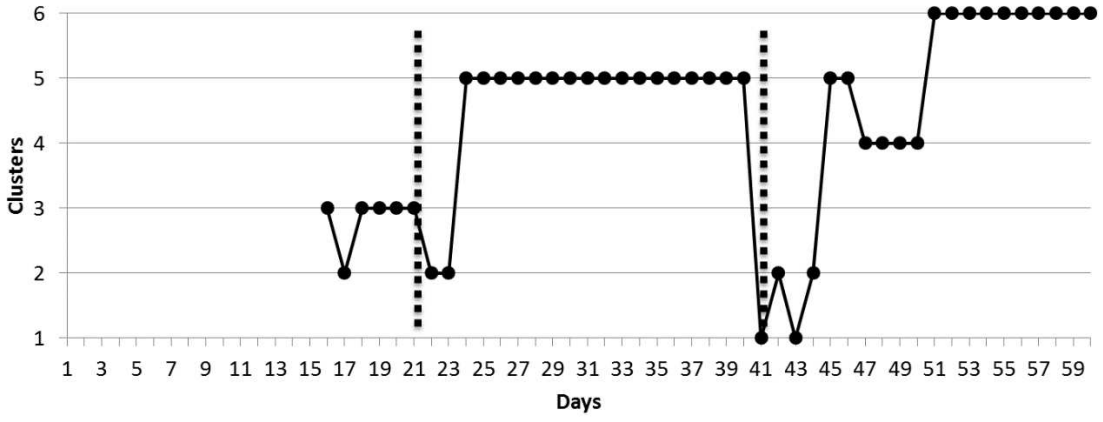

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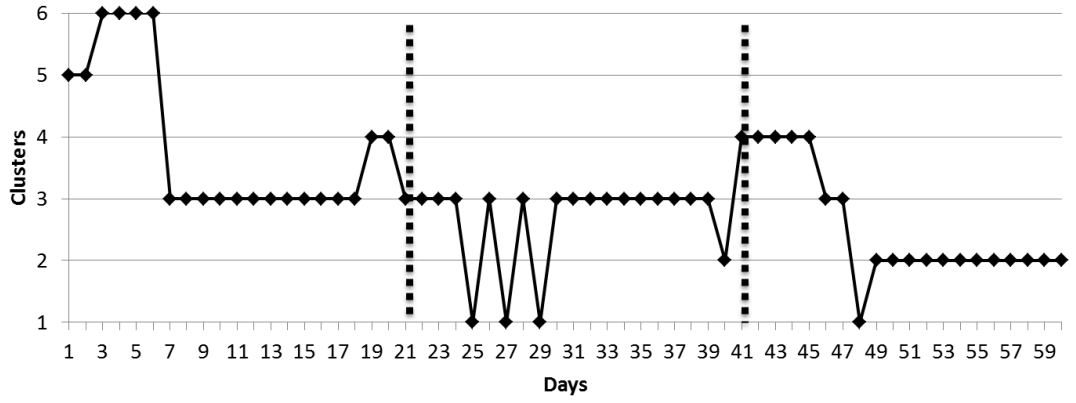
Figure 4



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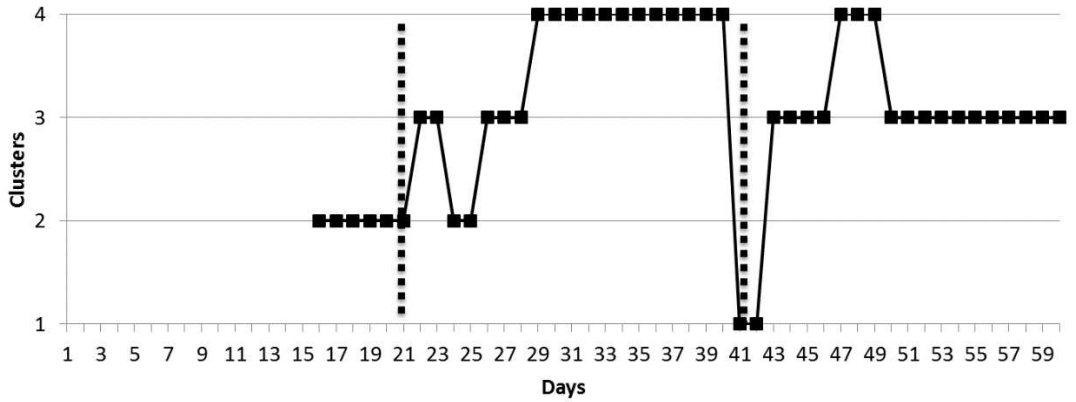

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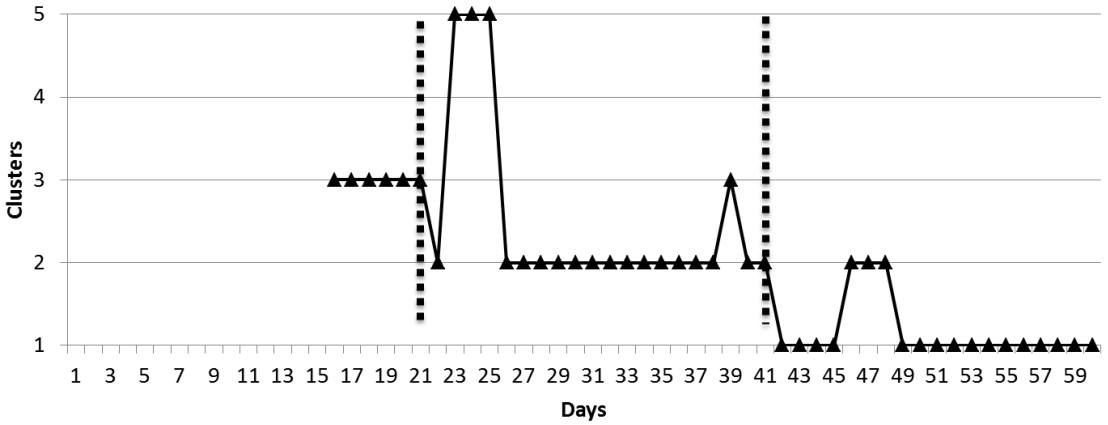

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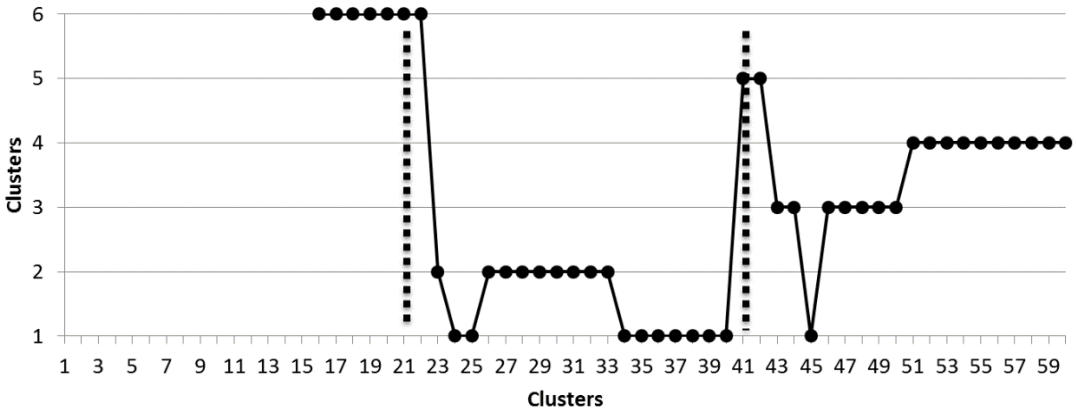

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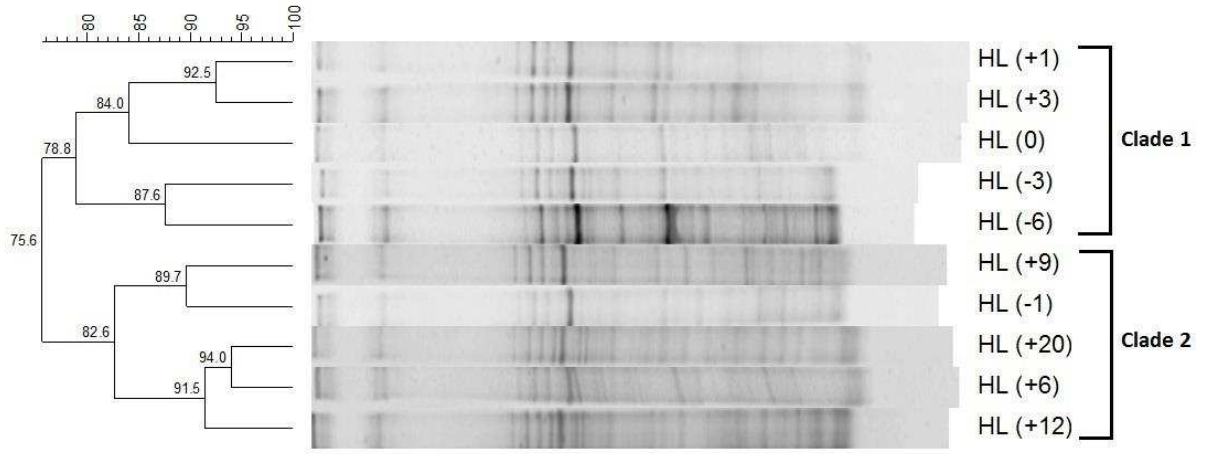
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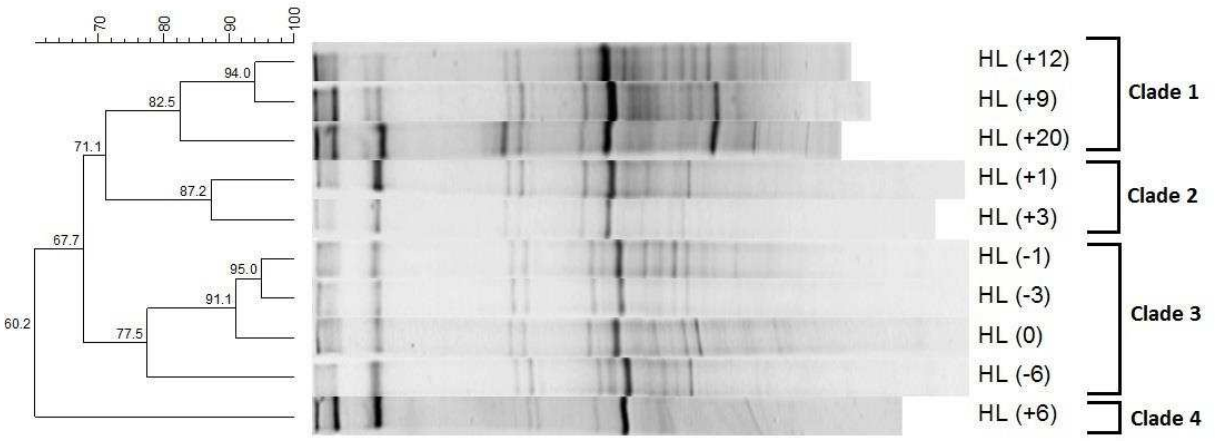
(a)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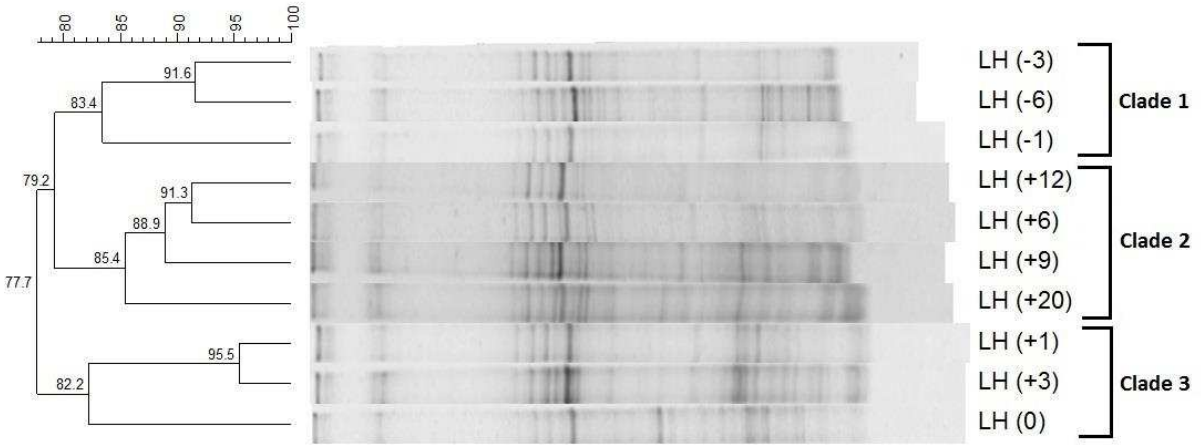
Clade 3
Clade 4

(b)

Figure 6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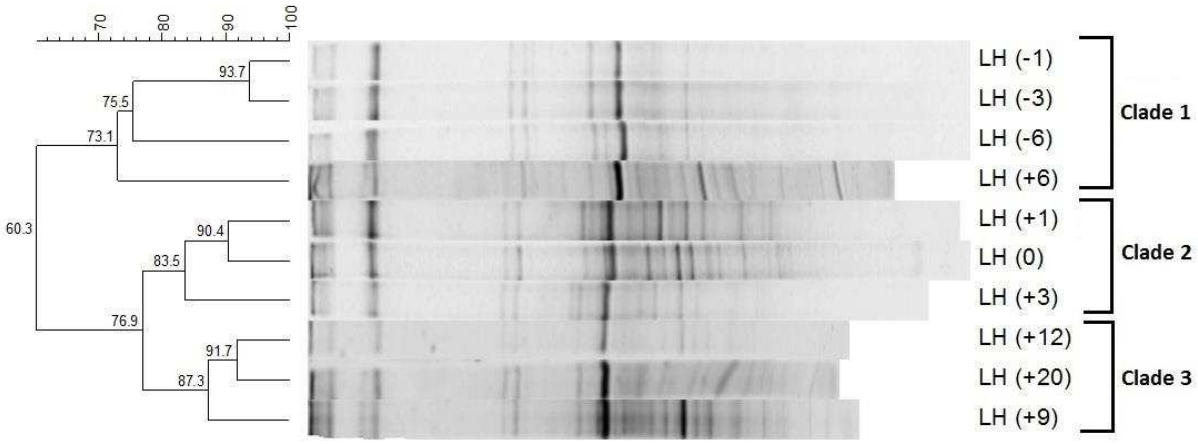
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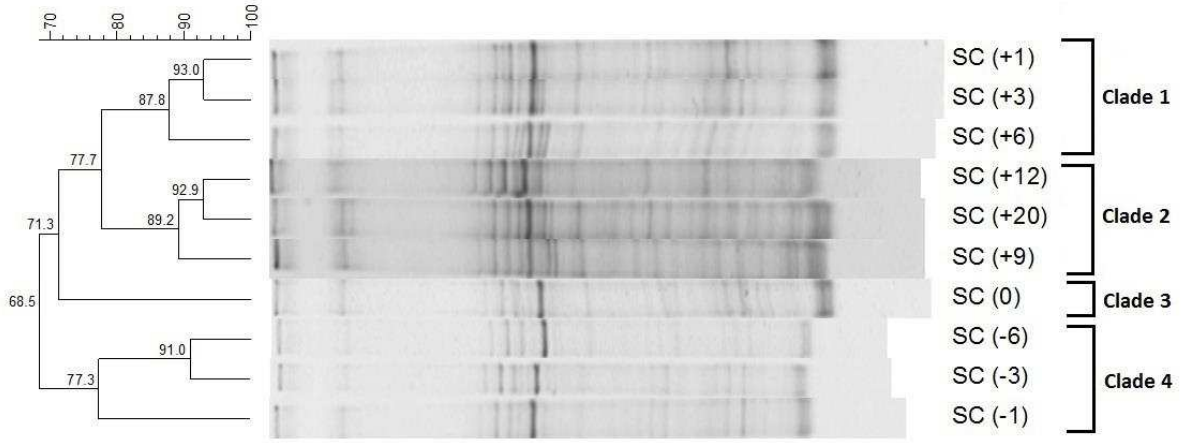
(b)

Figure 7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Clade 3
Clade 4

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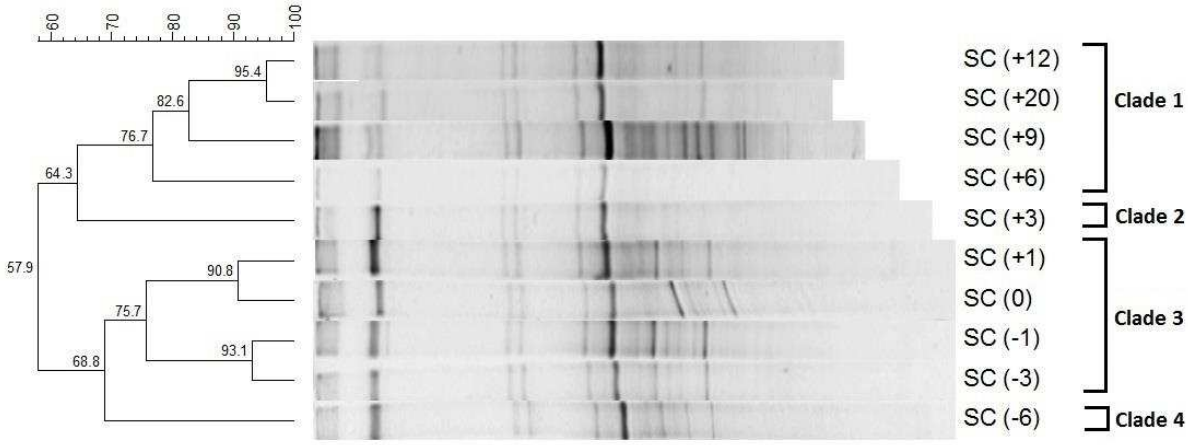
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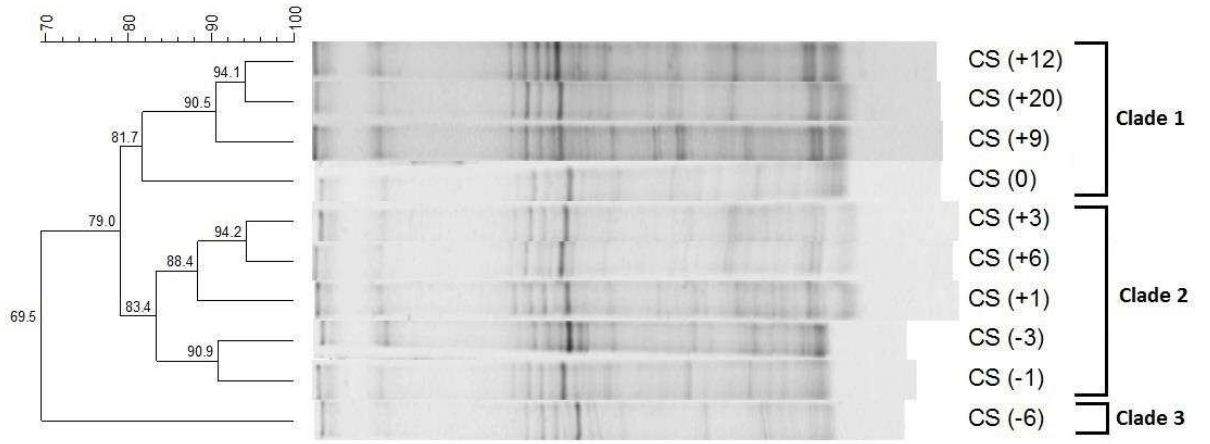
Clade 4

(b)

Figure 8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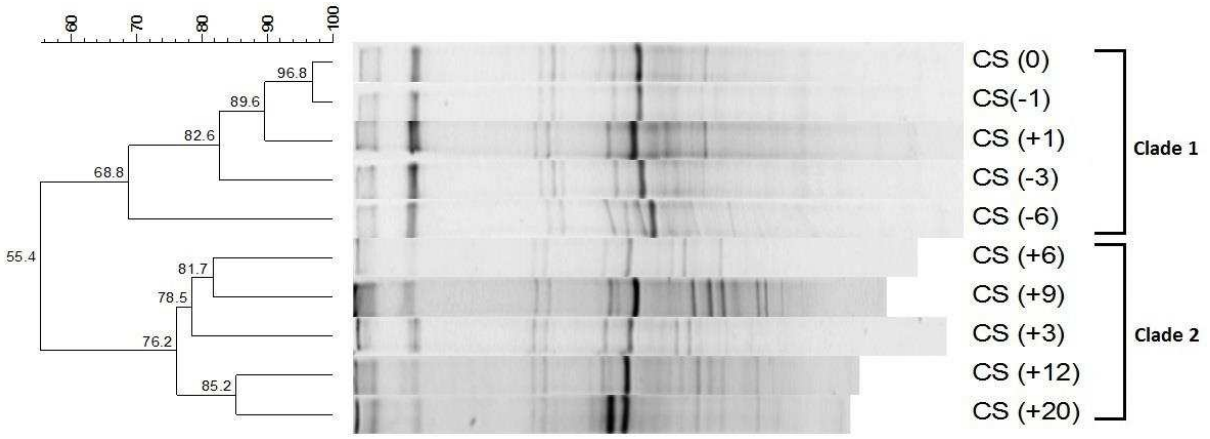
Clade 3

(a)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Clade 2

(b)

APPENDIX

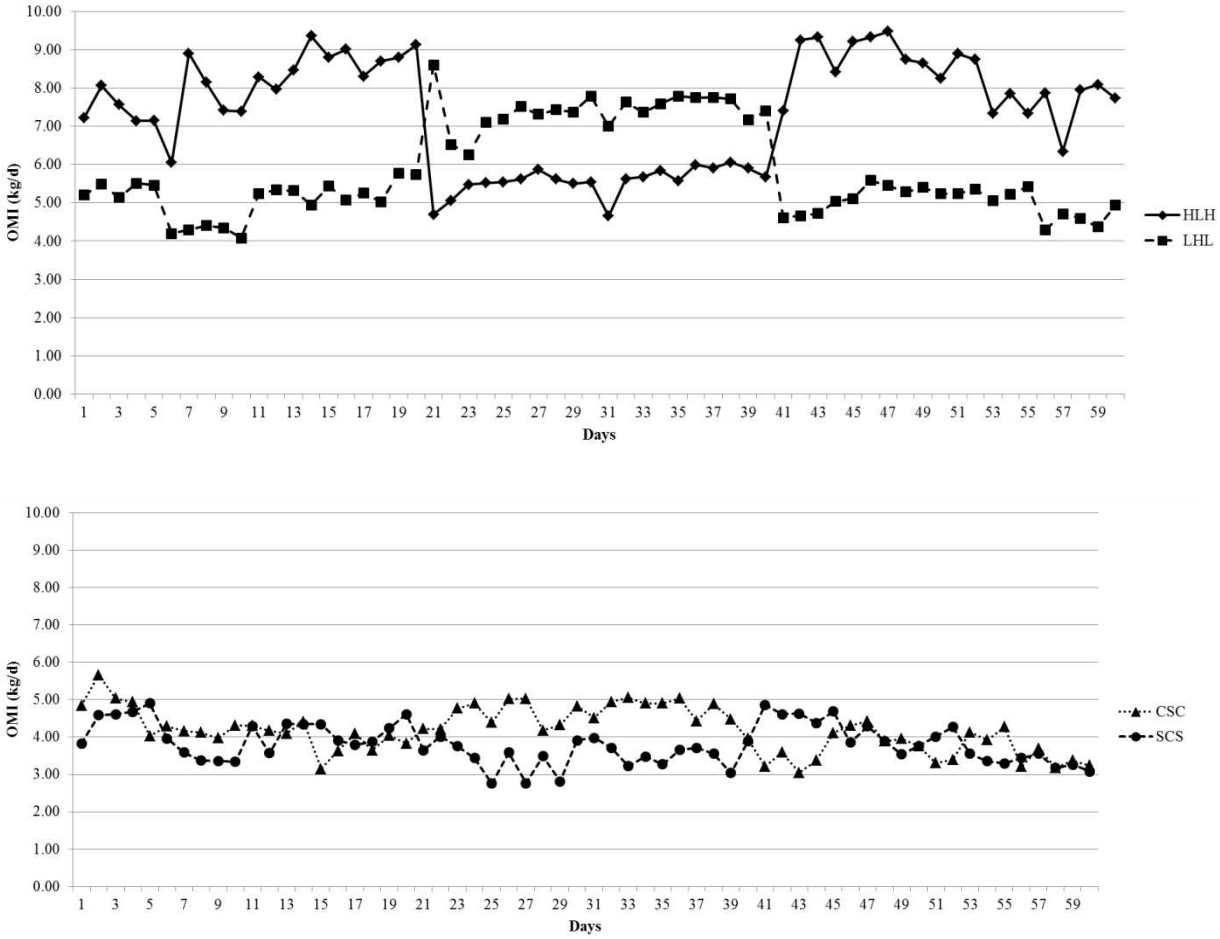

Figure 1A. Least square means for organic matter intake (OMI, kg/d) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

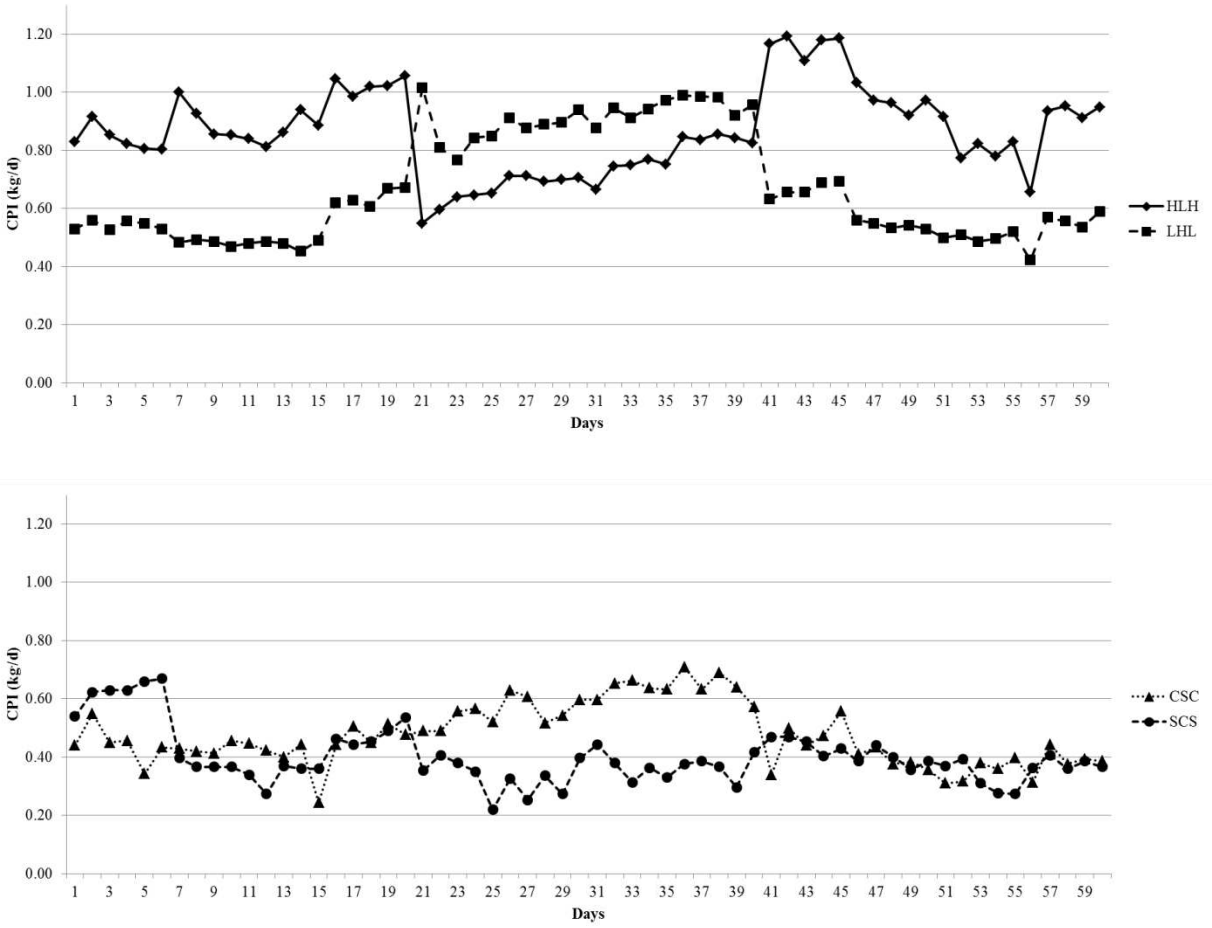

Figure 2A. Least square means for crude protein intake (CPI, kg/d) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

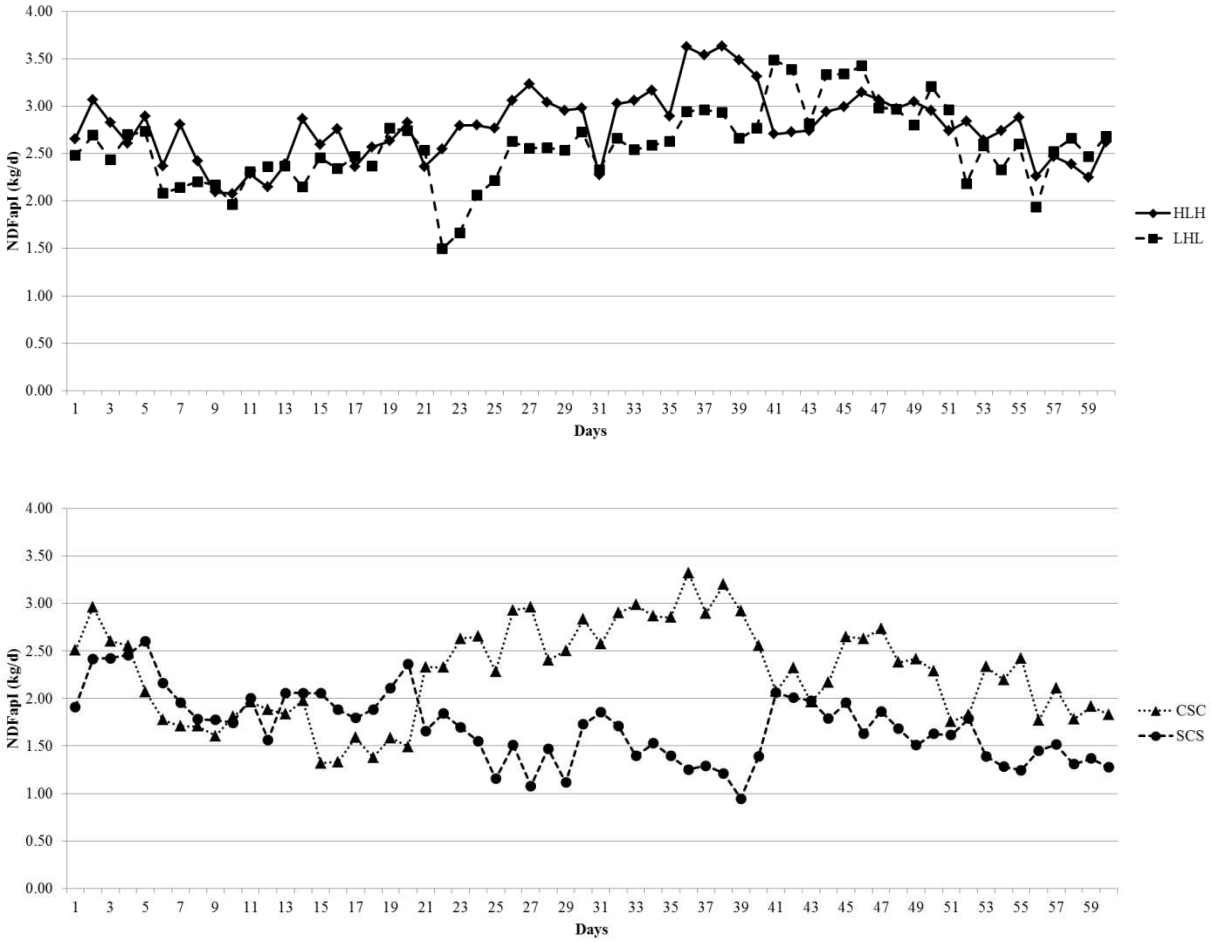

Figure 3A. Least square means for neutral detergent fiber corrected for ashes and protein intake (NDFapI, kg/d) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

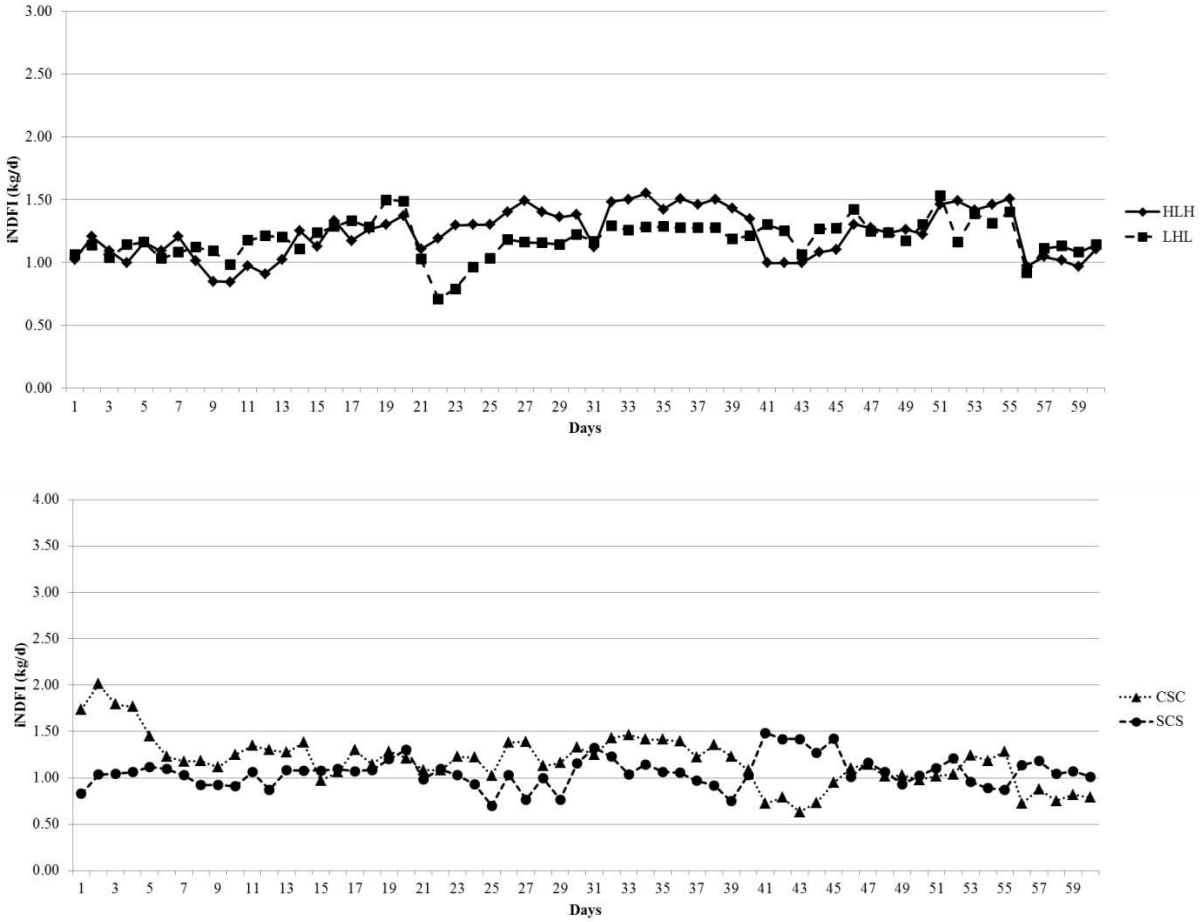

Figure 4A. Least square means for indigestible neutral detergent fiber intake (iNDFI, kg/d) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

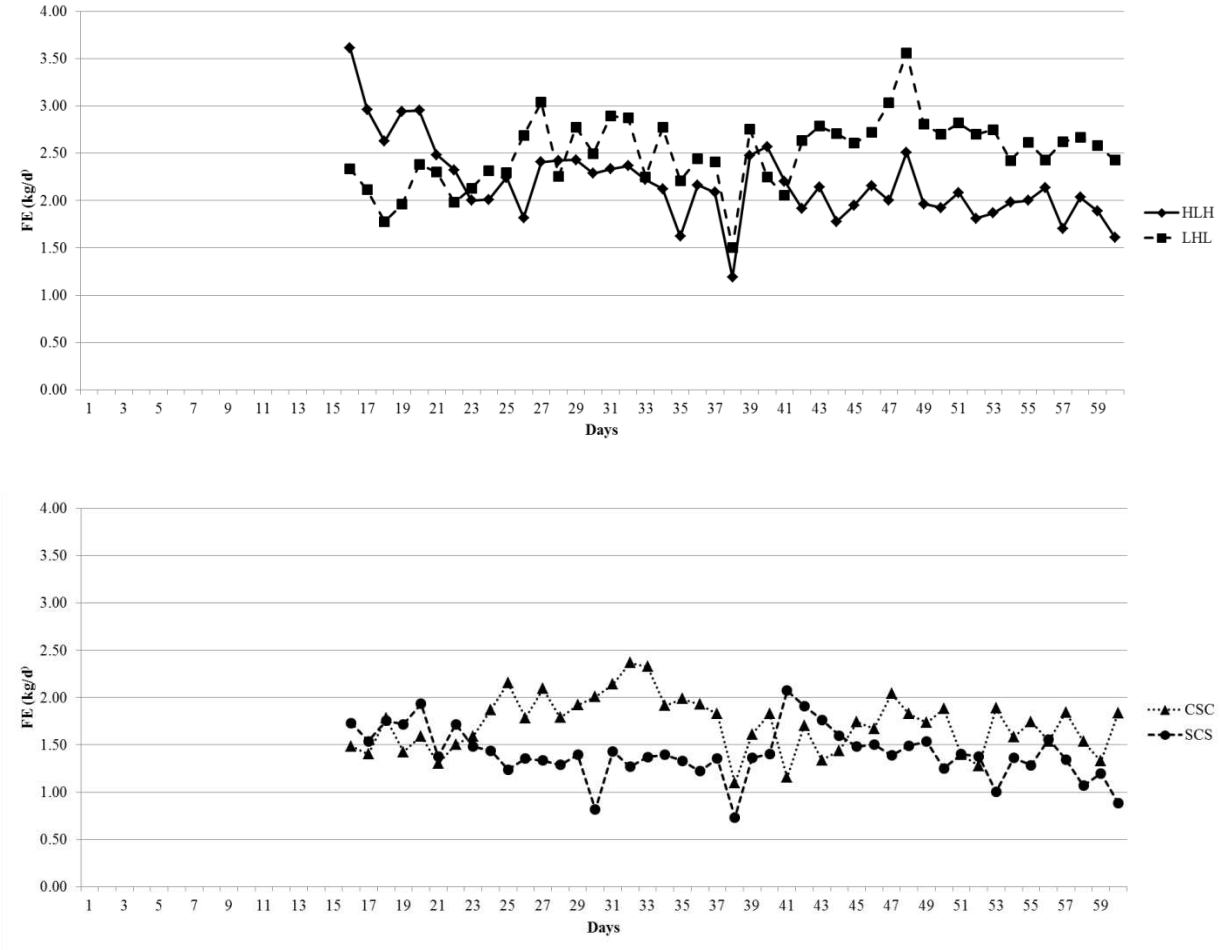

Figure 5A. Least square means for DM fecal excretion (EF, kg/d) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

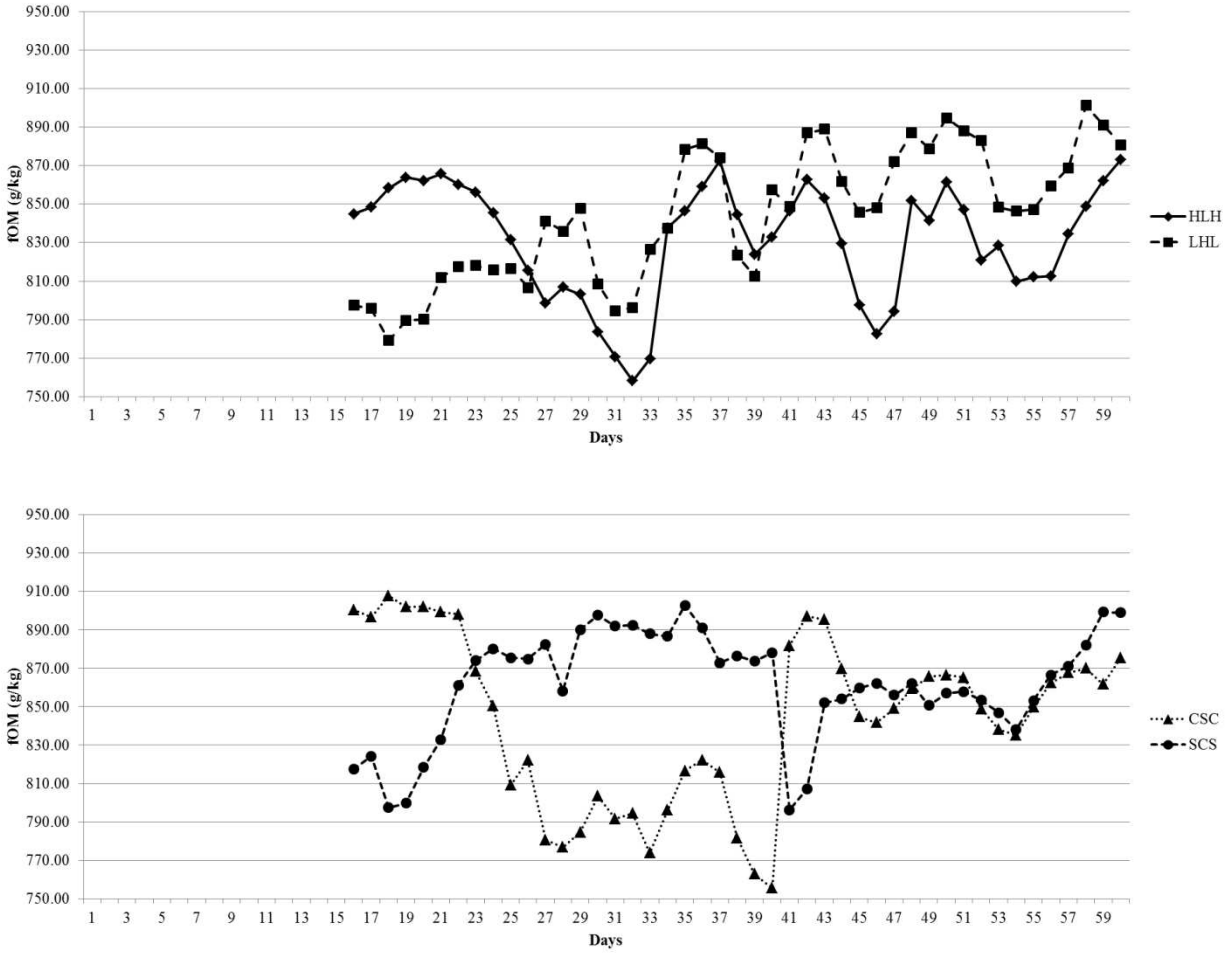

Figure 6A. Least square means for fecal organic matter (fOM, g/kg DM) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

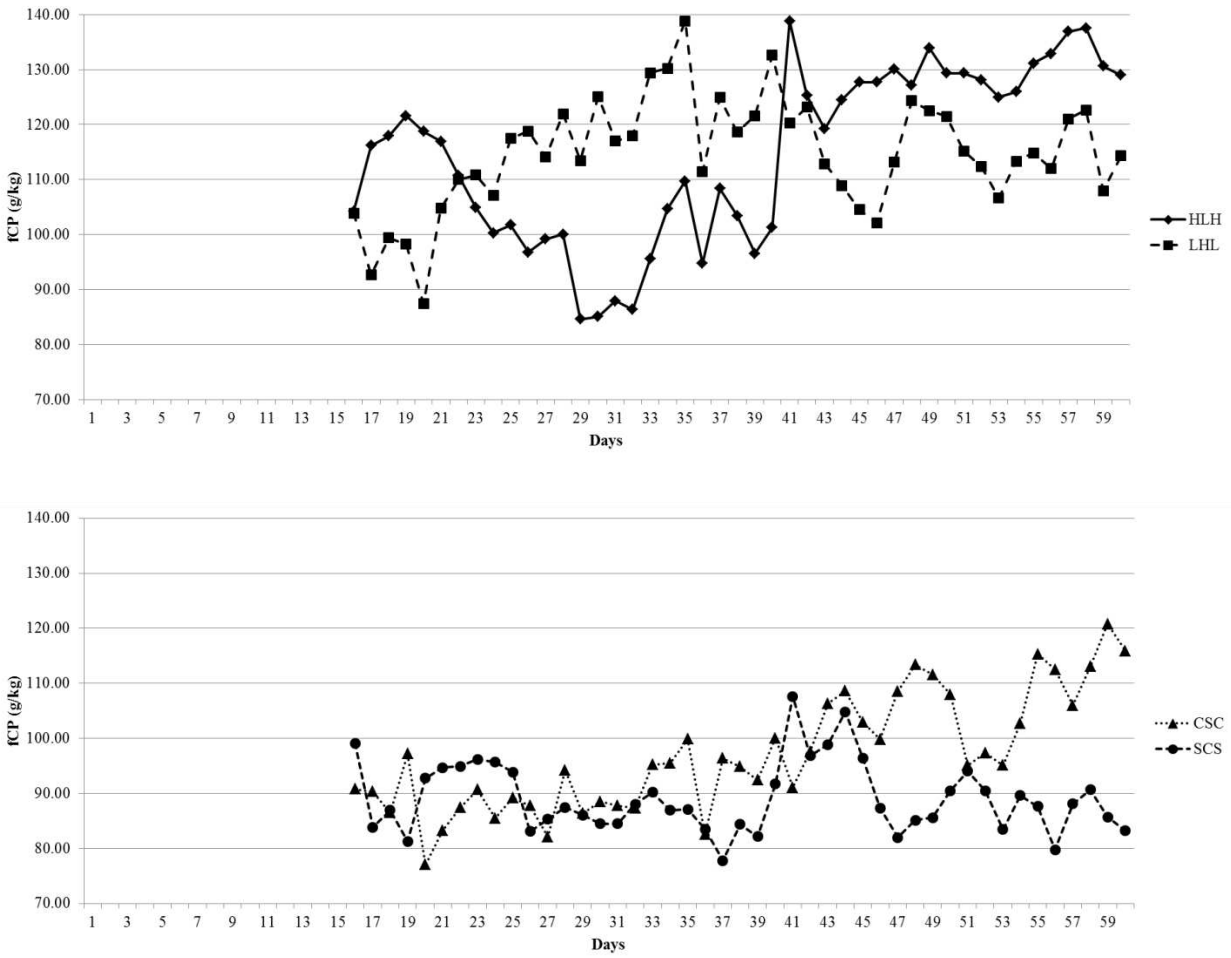

Figure 7A. Least square means for fecal crude protein (fCP, g/kg DM) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

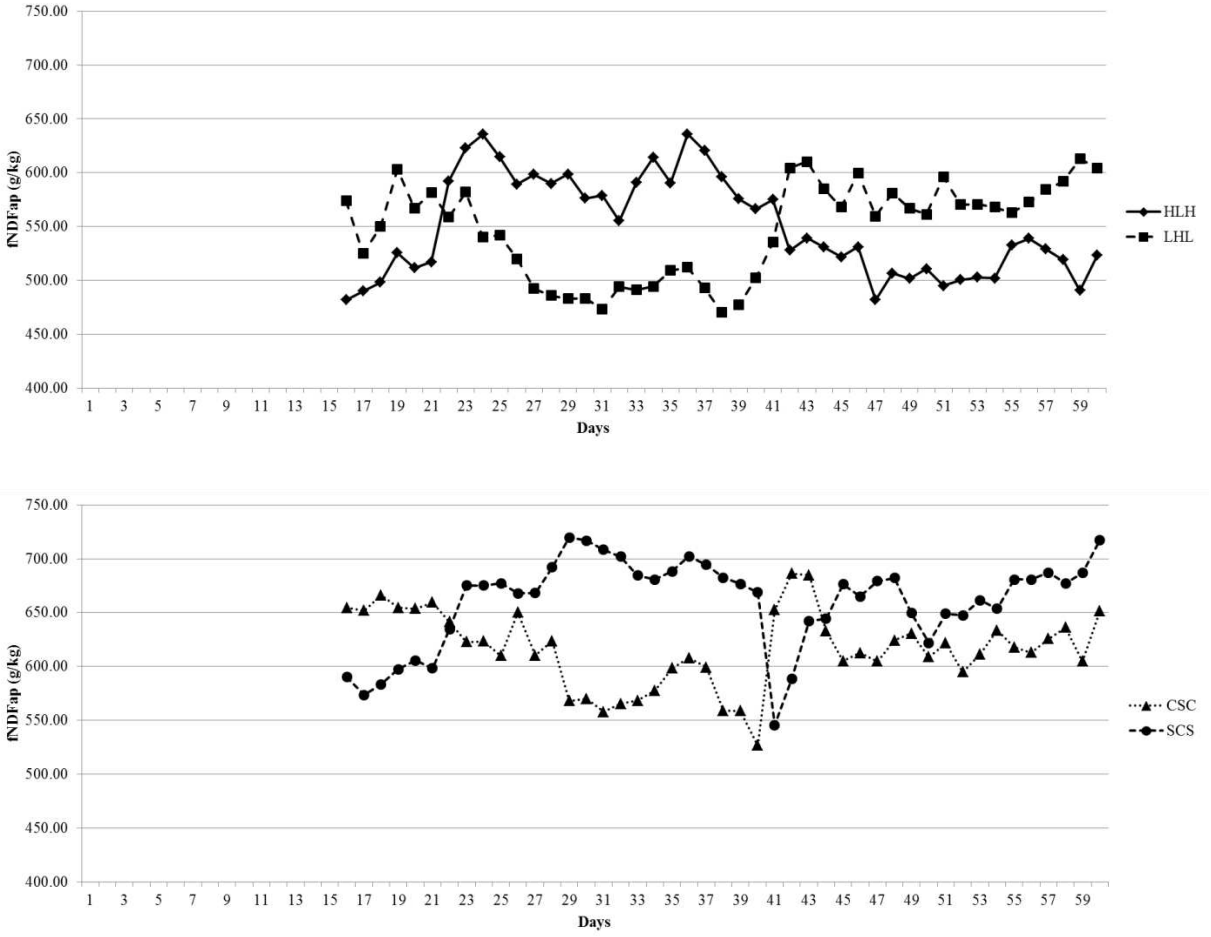

Figure 8A. Least square means for fecal neutral detergent fiber corrected for ashes and protein (fNDFap, g/kg DM) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage- sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

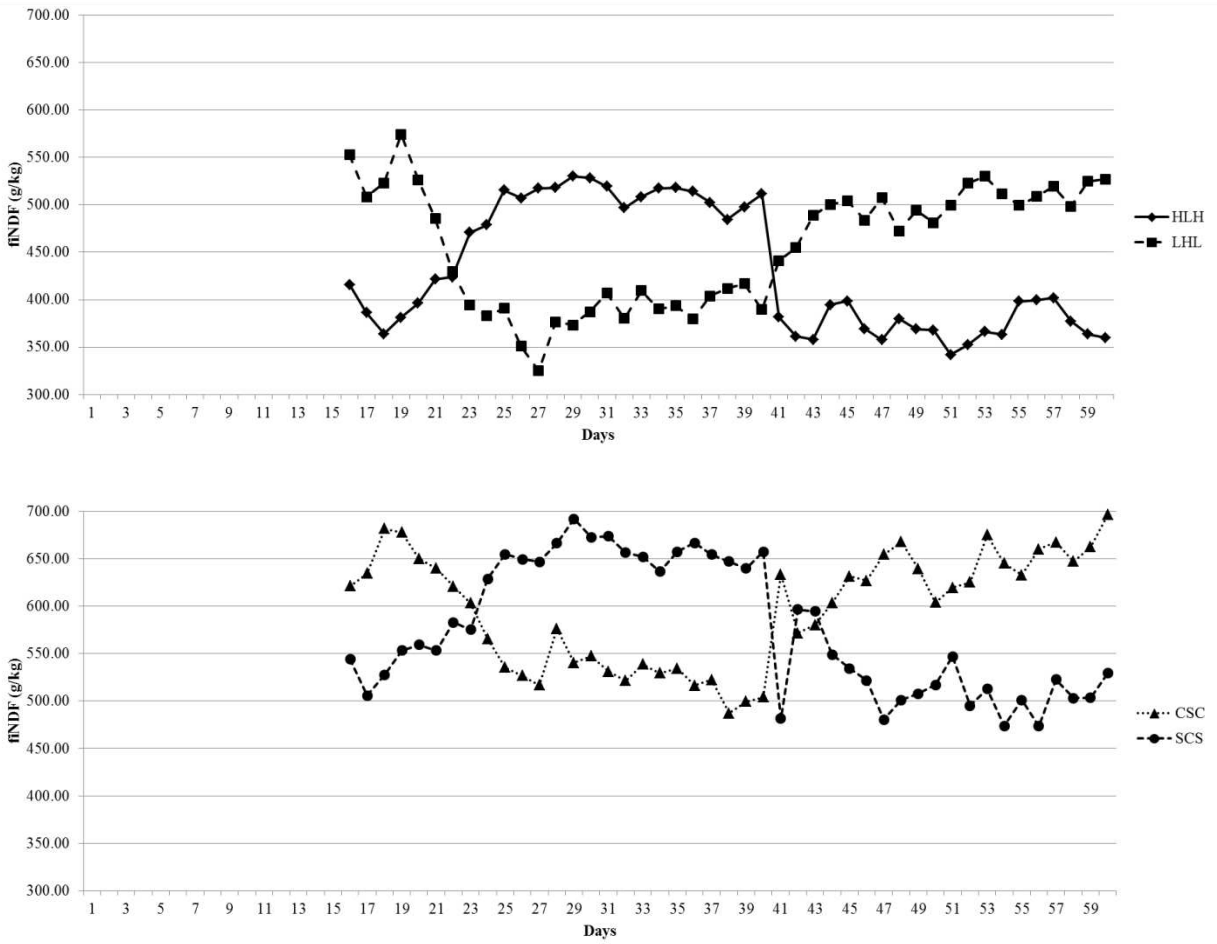

Figure 9A. Least square means for fecal indigestible neutral detergent fiber (fiNDF, g/kg DM) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

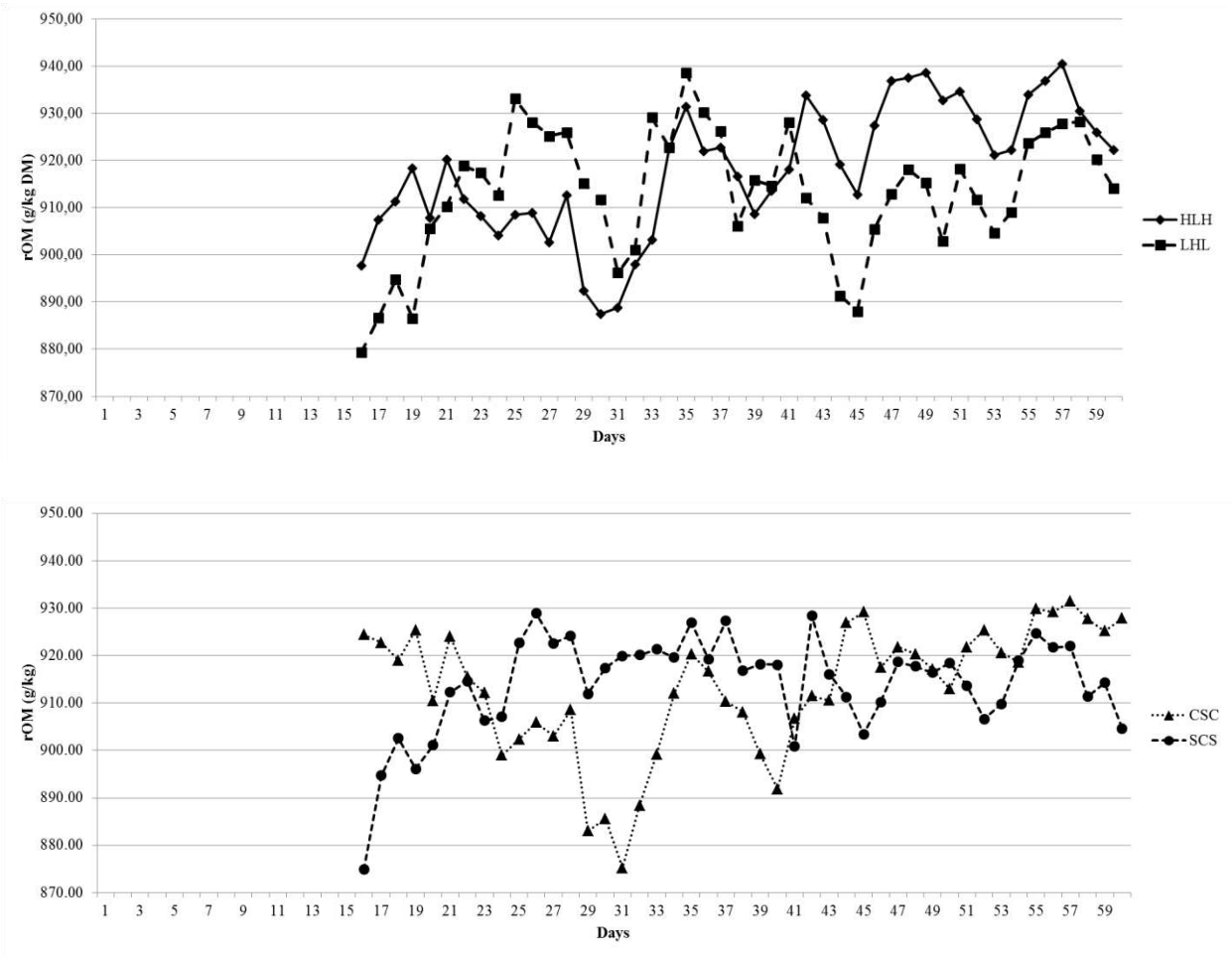

Figure 10A. Least square means for ruminal organic matter (rOM, g/kg DM) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

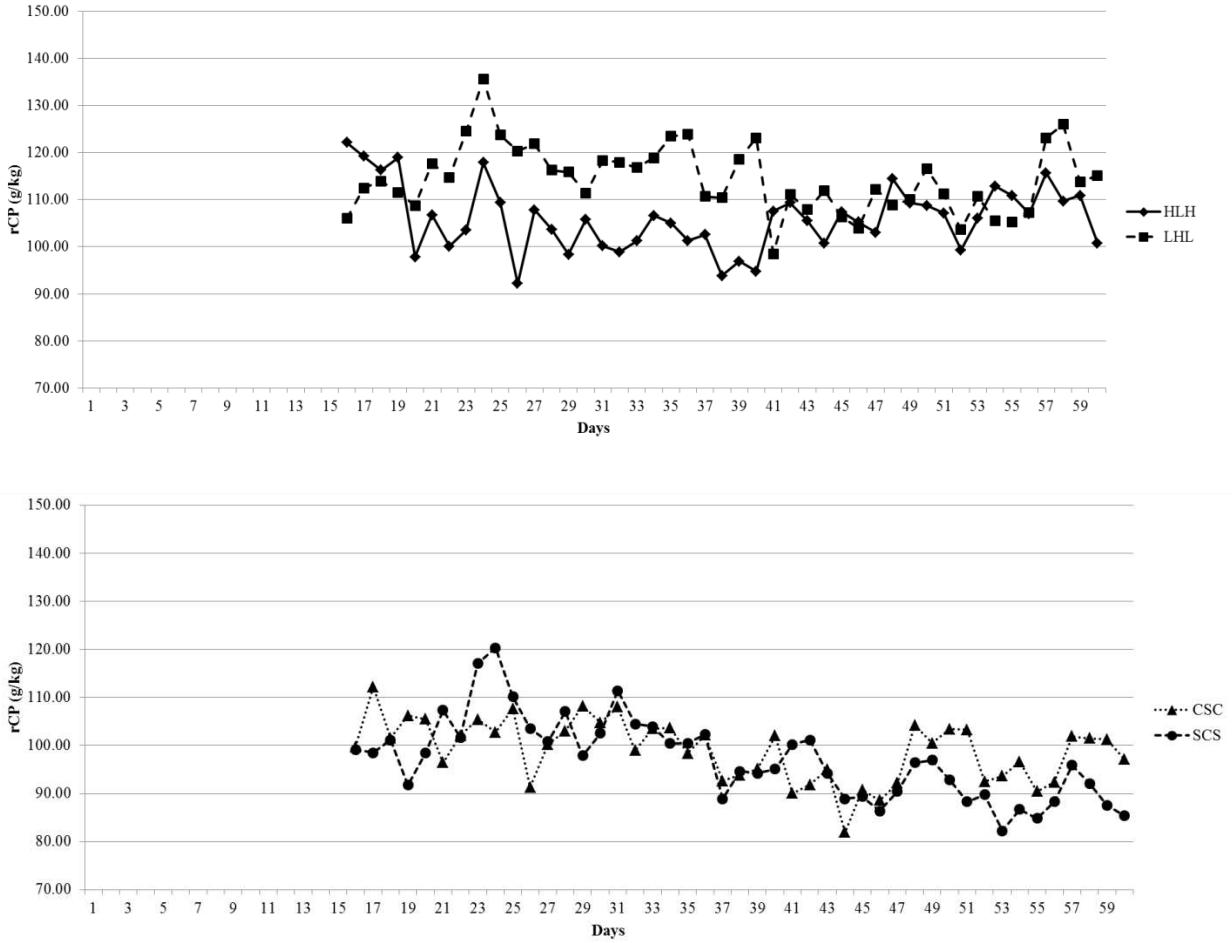

Figure 11A. Least square means for ruminal crude protein (rCP, g/kg DM) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

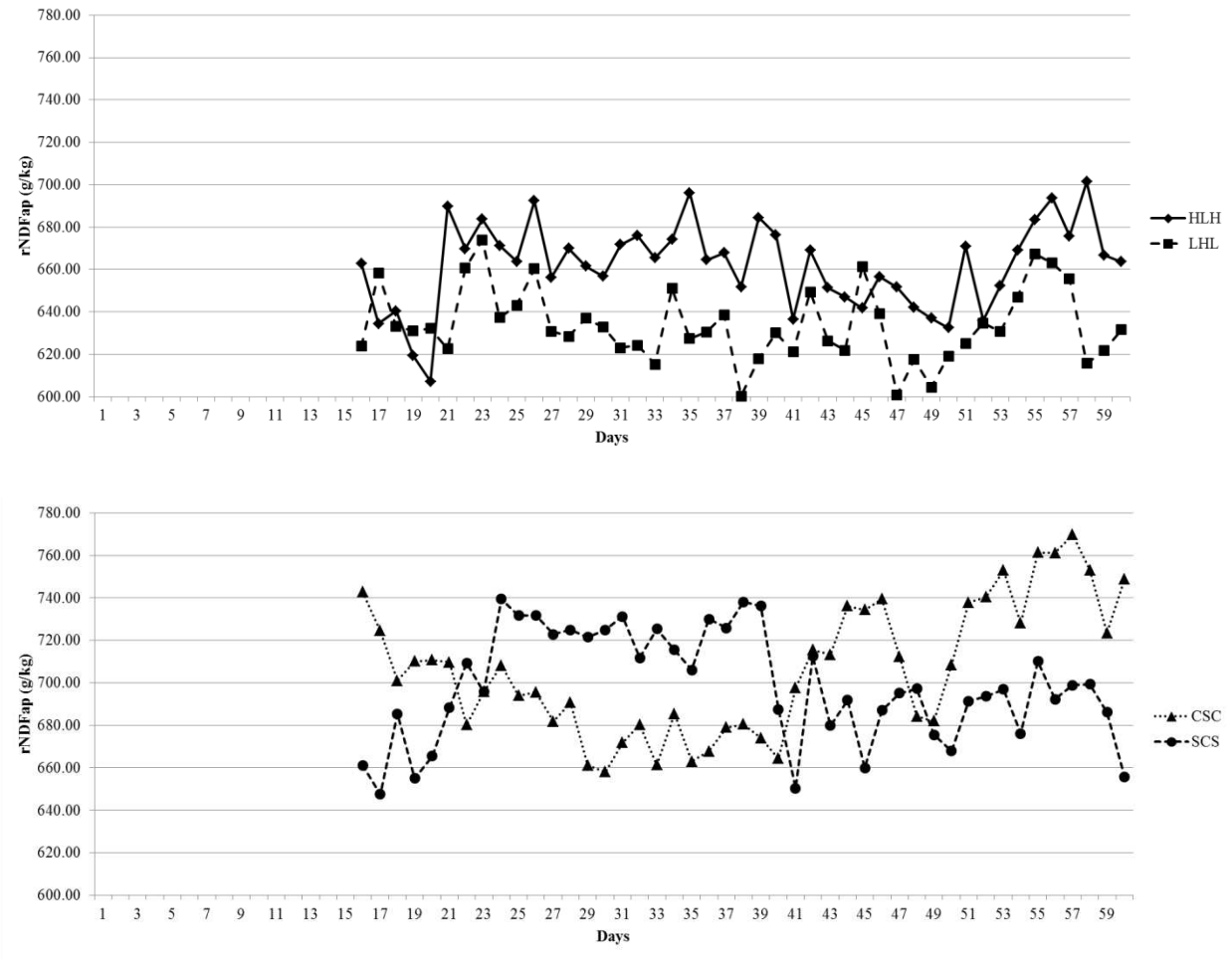

Figure 12A. Least square means for ruminal neutral detergent fiber corrected for ashes and protein (rNDFap, g/kg DM) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

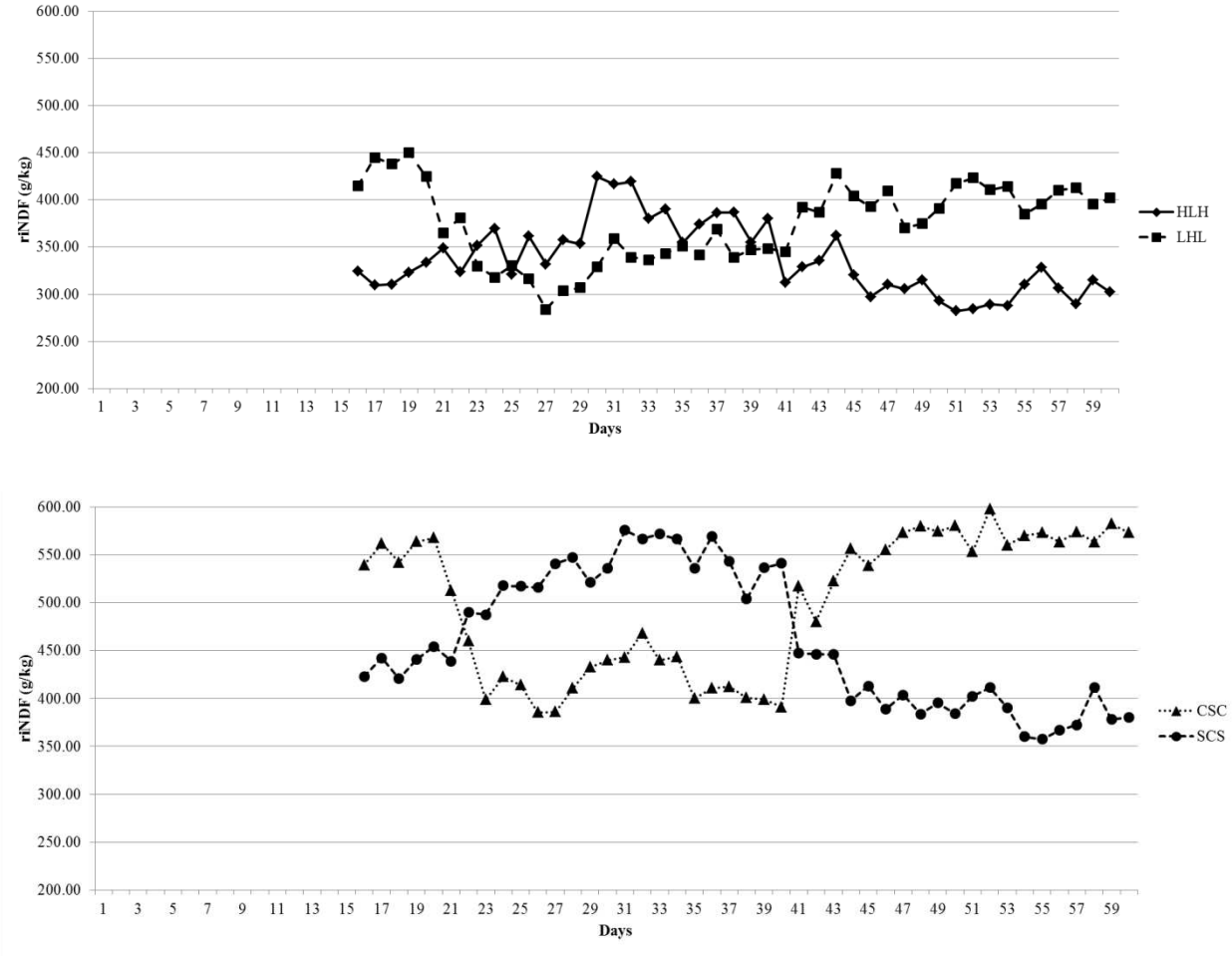

Figure 13A. Least square means for ruminal indigestible neutral detergent fiber (riNDF, g/kg DM) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

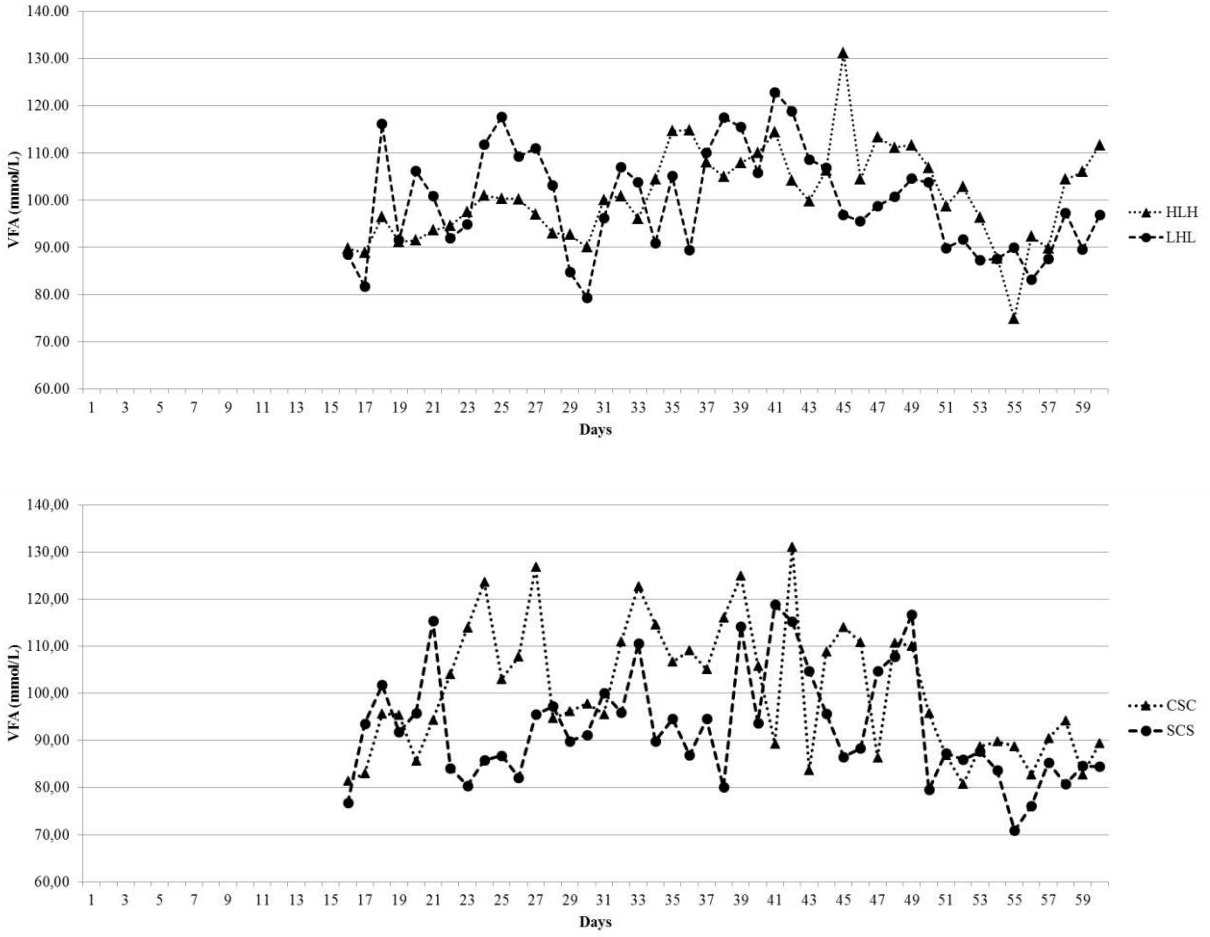

Figure 14A. Least square means for VFA concentration in rumen fluid (mmol/L) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage-sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

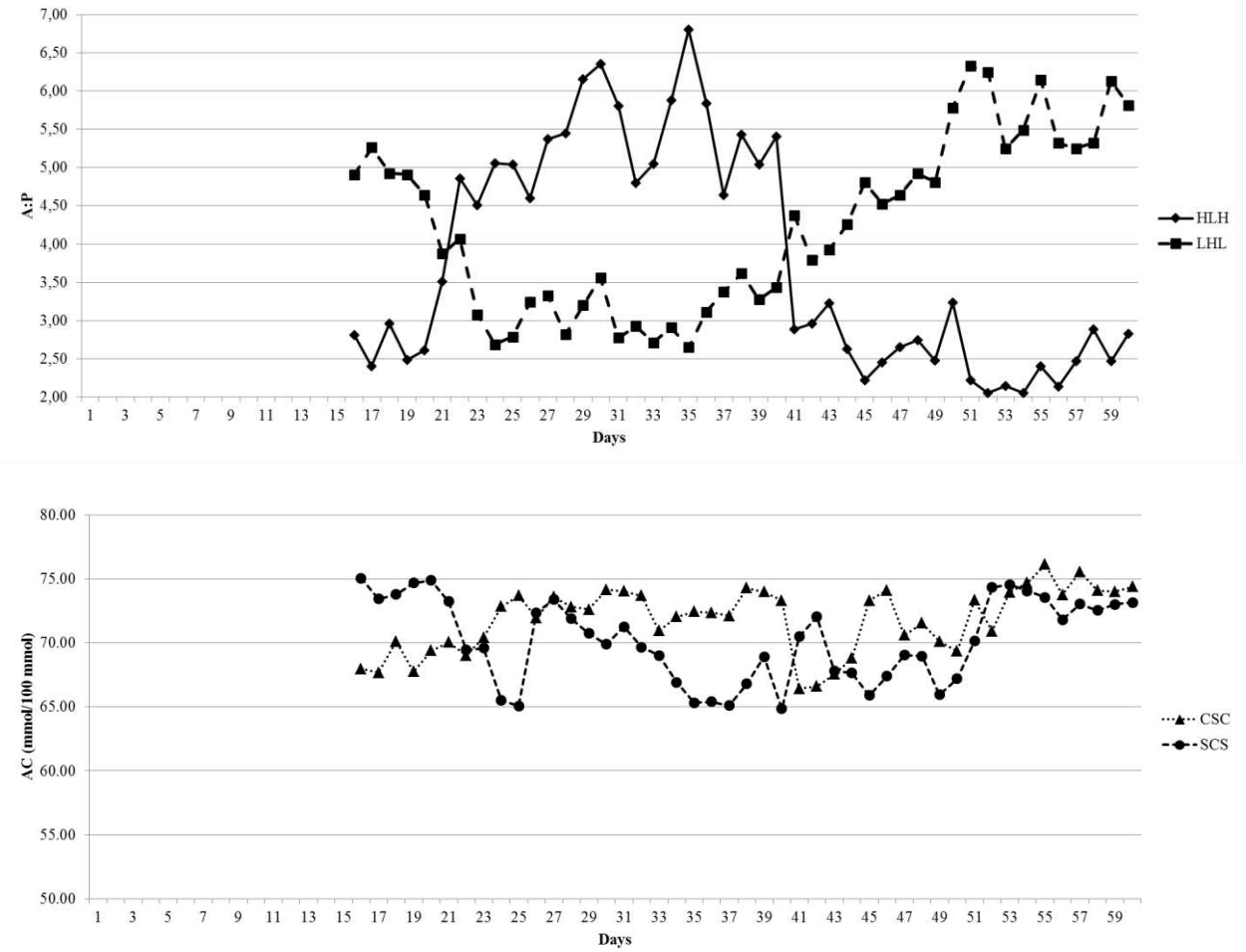

Figure 15A. Least square means for molar proportion of acetate in the rumen fluid (AC, mmol/100 mmol VFA) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage- sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

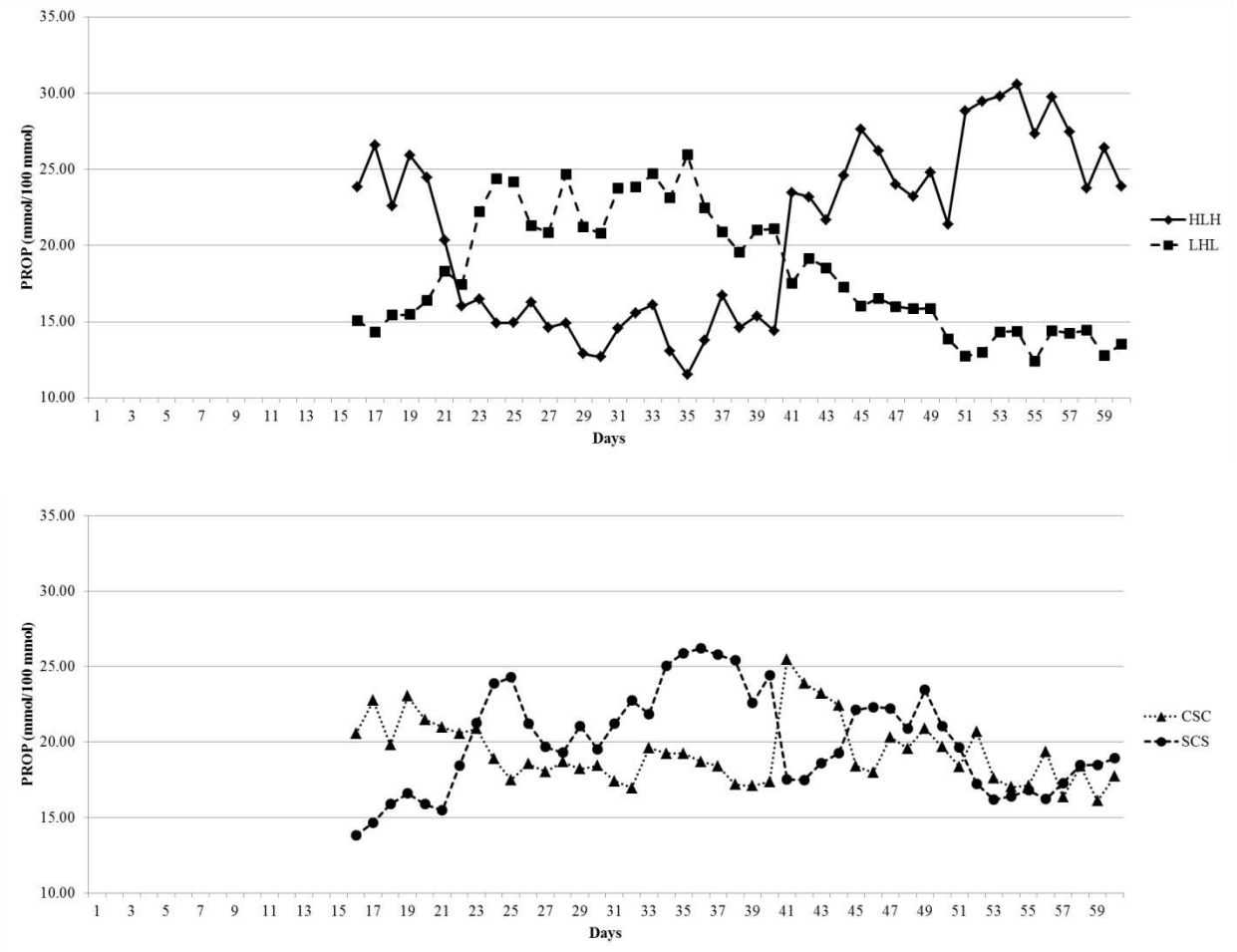

Figure 16A. Least square means for molar proportion of propionate in the rumen fluid (PROP, mmol/100 mmol VFA) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage- sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

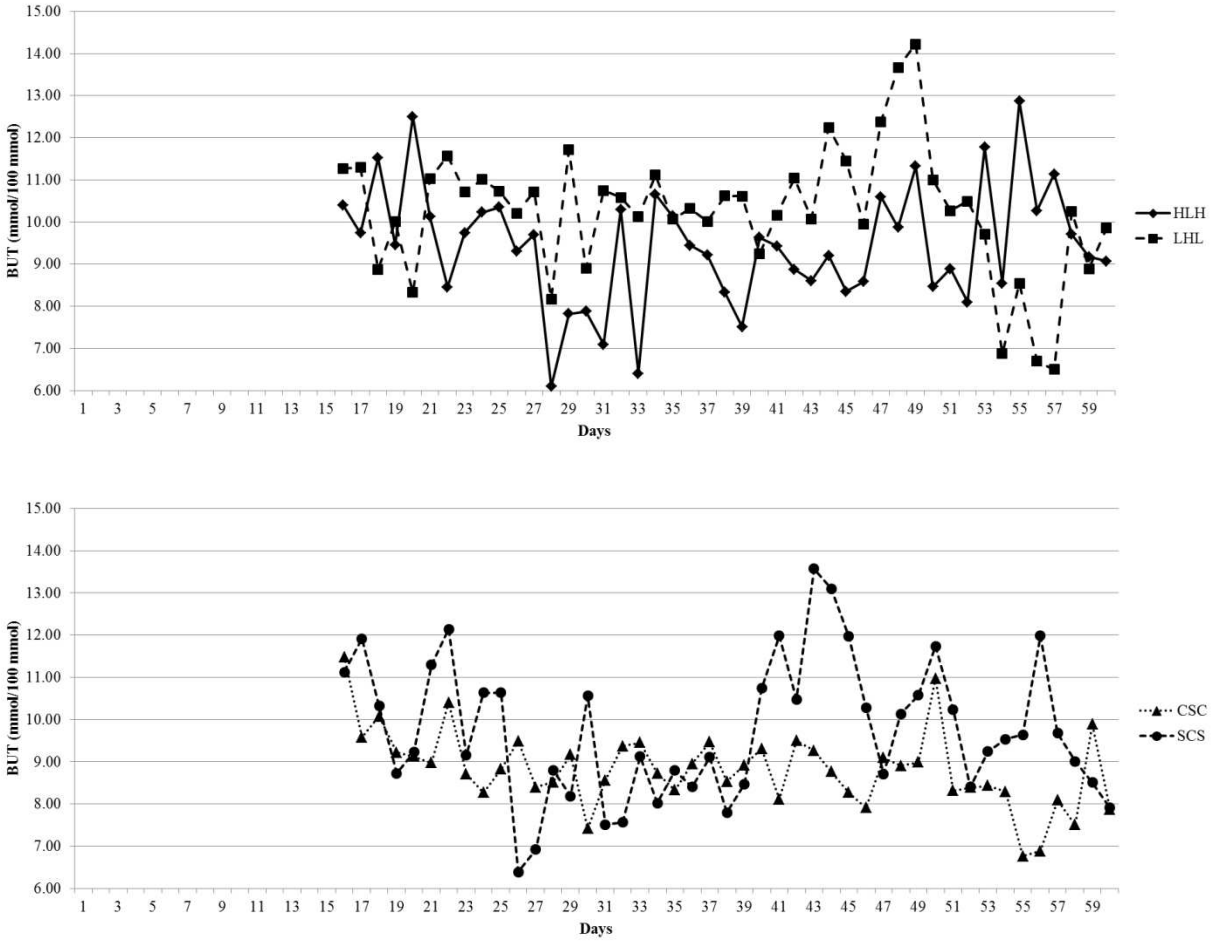

Figure 17A. Least square means for molar proportion of butyrate in the rumen fluid (BUT, mmol/100 mmol VFA) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage- sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

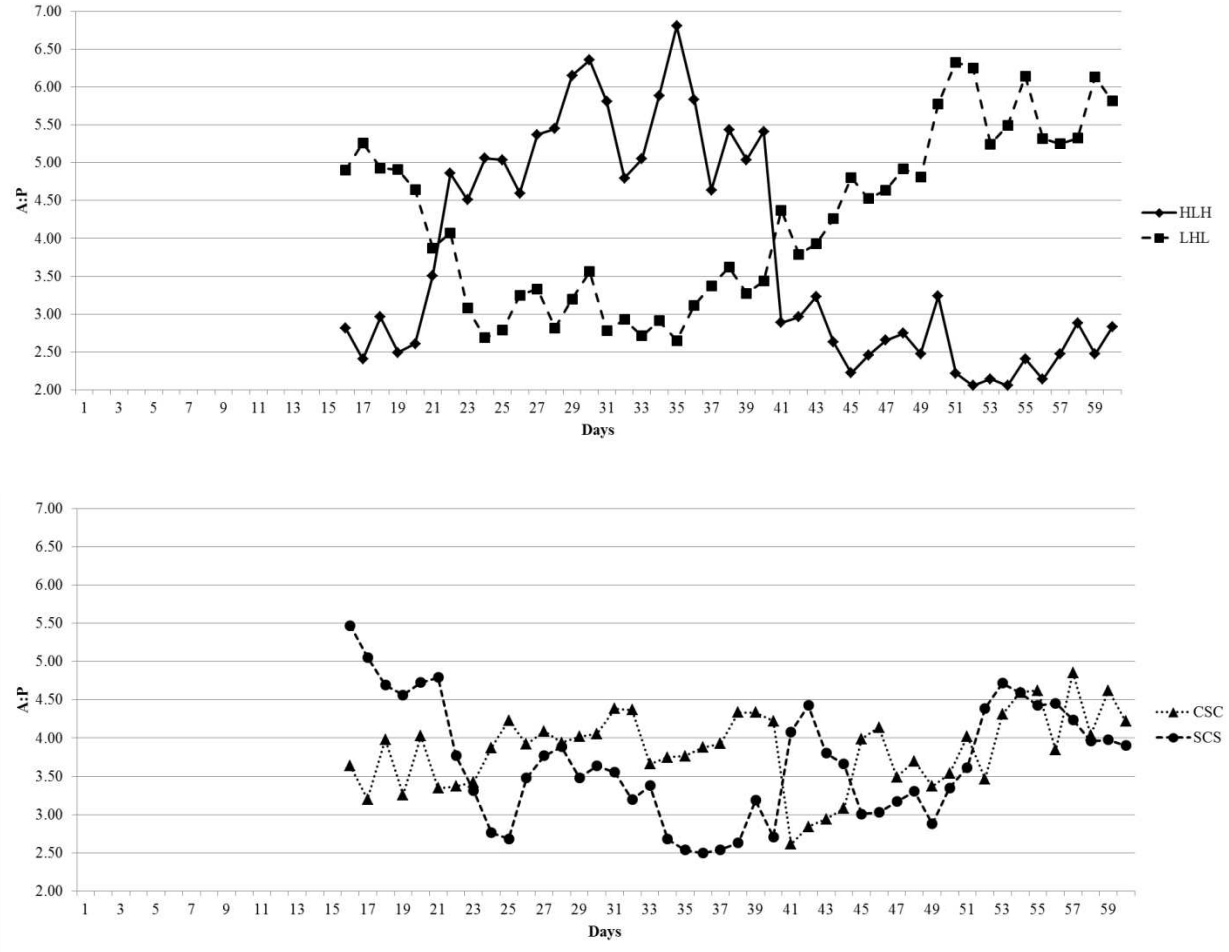

Figure 18A. Least square means for acetate to propionate ratio in the rumen fluid (A:P) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage- sugarcane-corn silage. Diet changes occurred on 21st and 41st days.

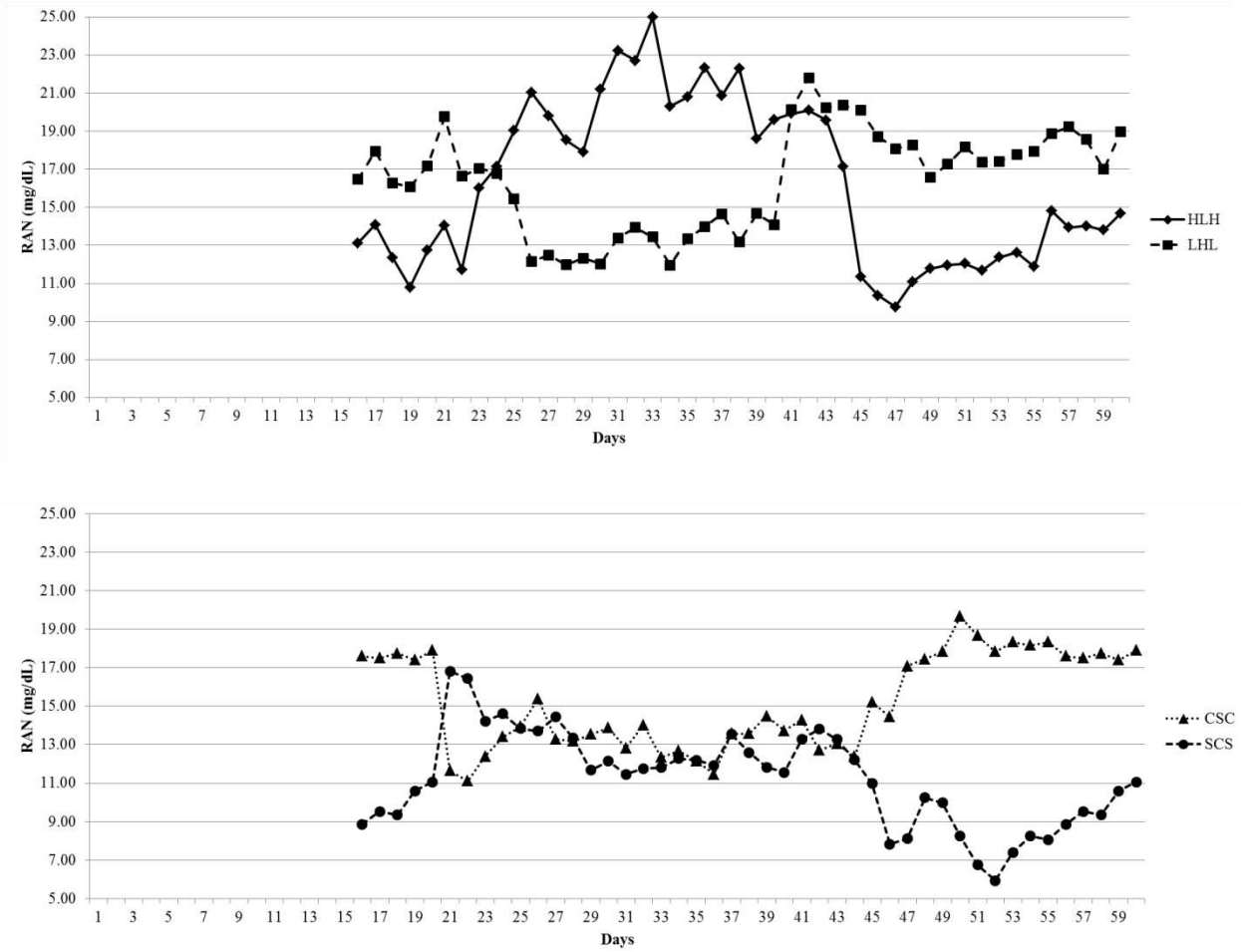

Figure 19A. Least square means for ruminal ammonia nitrogen concentration (RAN, mg/dL) for the different diet sequences according to the day of evaluation. HLH, sequence high concentrate-low concentrate-high concentrate; LHL, sequence low concentrate-high concentrate-low concentrate; CSC, sequence sugarcane-corn silage-sugarcane; SCS, sequence corn silage- sugarcane-corn silage. Diet changes occurred on 21st and 41st days.