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Journal of Dairy Research

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Research Article

Cite this article: Popova M, Ferlay A, Bougouin A, Eugène M, Martin C and Morgavi DP. Associating changes in the bacterial community of rumen and faeces and milk fatty acid profiles in dairy cows fed high-starch or starch and oil-supplemented diets. *Journal of Dairy Research* https://doi.org/10.1017/S0022029922000498

Received: 12 April 2022
Accepted: 26 April 2022

Keywords:
Bacterial communities; faeces; milk fatty acid profile; rumen

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Abstract

The experiment reported in this research paper aimed to evaluate the effects of high-starch or starch and oil-supplemented diets on rumen and faecal bacteria, and explore links between the structure of bacterial communities and milk fatty acid (FA) profiles. We used four Holstein dairy cows in a 4 × 4 Latin square design. Cows were fed a diet rich in cereals (high-starch diet with 23% starch content on dry matter (DM) basis), a diet supplemented with saturated FA from Ca salts of palm oil + 18% DM starch, a diet with high content of monounsaturated FA (from extruded rapeseeds) + 18% DM starch or a diet rich in polyunsaturated FA (from extruded sunflower seeds) + 17% DM starch. At the end of each experimental period, cows were sampled for rumen and faecal contents, which were used for DNA extraction and amplicon sequencing. Partial least squares (PLS) regression analysis highlighted diet-related changes in both rumen and faecal bacterial structures. Sparse PLS discriminant analysis was further employed to identify biologically relevant operational taxonomical units (OTUs) driving these differences. Our results show that *Butyrivibrio* discriminated the high-starch diet and linked positively with higher concentrations of milk odd- and branched-chain FA. YS2-related OTUs were key taxa distinguishing diets supplemented with Ca salts of palm oil or sunflower seeds and correlated positively with linoleic acid in milk. Similarly, diets modulated faecal bacterial composition. However, correlations between changes in faecal and rumen bacteria were poor. With this work, we demonstrated that high-starch or lipid-supplemented diets affect rumen and faecal bacterial community structure, and these changes could have a knock-on effect on milk FA profiles.

Dairy products are a source of many micro- (calcium, phosphorus, vitamins) and macronutrients (lipids, proteins, sugars) (Pereira, 2014). Among lipids, the potential beneficial effect of polyunsaturated fatty acids (PUFA), notably conjugated linoleic acids (CLA), is known (Ferlay *et al.*, 2017). Besides, the pentadecanoic ($C_{15}:0$) and heptadecanoic ($C_{17}:0$) acids showed anticarcinogenic effects in *in vitro* and *in vivo* studies (Bernard *et al.*, 2018). On the one hand, milk consumption has been associated with a decreased risk of vascular diseases (Givens, 2010), whereas dairy fat consumption positively correlated with unhealthful saturated fatty acids (SFA) in plasma (Jenkins *et al.*, 2015). For many years, attention was also drawn to *trans* fatty acids (FA) involved in cardiovascular diseases. However, it appears that milk *trans* FA have a less negative impact than industrial fats (Ferlay *et al.*, 2017). Regardless, *trans* FA and SFA have been a concern to consumers, and prevailing dietary guidelines recommend keeping low intake of SFA and replacing SFA with mono-unsaturated fatty acids (MUFA) or PUFA (FAO, 2010).

Milk *de novo* FA are synthesised in the mammary gland from volatile fatty acids (VFA) produced by rumen microbes or modified by a delta9-desaturase. Rumen microbes synthesise FA with an odd number of carbons ($C_{15}:0$, $C_{17}:0$) as components of their lipid membrane but could also produce CLA isomers through partial biohydrogenation processes of dietary PUFA. Therefore, researchers have already suggested the potential role of rumen microbiota in modulating fat profiles in cow milk (Vlaeminck *et al.*, 2006; Jami *et al.*, 2014; Ferlay *et al.*, 2017). Recently, Stergiadis *et al.* (2021) compared the rumen microbial community structure of cows fed the same diet but producing high or low levels of SFA in milk. They reported a positive correlation between milk SFA and microbial abundance of lactic acid bacteria, Proteobacteria, Actinobacteria and anaerobic fungi. Though these inter-individual variations could be explained by the heritability and microbiability of the animal phenotype (Huws *et al.*, 2018; Buitenhuis *et al.*, 2019), diet has been acknowledged as a primary factor affecting microbial community structure and animal performance (Huws *et al.*, 2018).

Supplementing diets with vegetal lipids improves dietary energy supply in lactating dairy cows and, compared to high-starch diets, this supplementation limits ruminal pH decrease and digestive acidosis disorder (Martin *et al.*, 2010). Oilseed-supplemented diets are known to increase milk concentrations of FA with 18 carbons, potentially having beneficial effects on human health, and decrease short- and medium-chain SFA (reviewed in Lourenco *et al.*, 2010; Ferlay *et al.*, 2017). Oilseed-supplemented diets are under investigation for their ability to modulate rumen microbiota and alter PUFA biohydrogenation in the rumen. *In vitro*, for instance, sunflower and linseed oils decreased the relative abundance of Clostridiales and Actinobacteria and increased Lactobacillales species accompanied by an increase in vaccenic acid and CLA formation (Vargas *et al.*, 2020). However, *in vivo* data linking rumen microbial profiles to milk FA is scarce for all domesticated ruminants. Moreover, dietary composition could affect the amount of organic matter leaving the rumen and reaching the hindgut. This has been reported for starch-rich diets, where higher amounts of starch escaping the rumen disappeared in the caecum (Huntington *et al.*, 2006). Higher FA intake could also shift the site of nutrient digestion from the rumen to the hindgut (Harvatine and Allen, 2006). Therefore, further investigation, integrating rumen and caecum microbiota, is required to understand better the link between diet and milk FA.

The present study explored the relationship between diet-driven changes in the rumen and faecal bacterial communities and milk FA profiles in dairy cows. We took advantage of our previously published work (Bougouin *et al.*, 2019) demonstrating that high-starch or starch and lipid-supplemented diets modified milk FA profiles. We hypothesised that diet-induced changes in milk FA profiles can be assigned to shifts in rumen bacterial community structure and that these alterations could be also reflected in faecal bacteria. We used Illumina amplicon sequencing targeting the bacterial 16S rRNA gene in the rumen and faecal contents. The subsequent statistical analysis highlighted diet-driven changes in bacterial communities structure (both in rumen and faeces) and established links with the concentration of FA in milk.

Material and methods

The experimental design was the same as that described by Bougouin *et al.* 2019 and followed the guidelines of the French Ministry of Agriculture for animal research and the applicable European Union guidelines and regulations on animal experiments (Directive 2010/63). The Auvergne regional ethics committee on animal experimentation approved the experiment (reference number 821-2015060811534198).

Animals, diets and experimental design

Four multiparous lactating Holstein cows (60 ± 10 d in milk) were used in a 4 × 4 Latin square design. Each experimental period lasted 28 d. During the first 20 d, cows were in a free stall barn. Then, from day 21 to 26 cows were placed in respiratory chambers for methane measures before being returned to the free stall barn for the last 2 d. The basal diet was composed (dry matter (DM) basis) of 56% maize silage, 4% hay and 40% pelleted concentrate. Diets differed in the nature of energy supplied by the concentrate rich in (1) starch from maize grain and wheat, (2) SFA from Ca salts of palm oil, (3) MUFA from extruded rapeseeds, and (4) PUFA from extruded sunflower seeds (online Supplementary Table 1). Hay was supplied once a day (0830 h), while maize silage and concentrates were mixed together and fed twice a day as a partial mixed ration (66% at 0900 h and 34% at 1600 h). Detailed information is provided in Bougouin *et al.* (2019).

Sample collection

Milk samples were collected at each milking on days 22–24; detailed sampling procedure and analyses were described in Bougouin *et al.* (2019). Briefly, lipids from milk samples were transmethylated, and the FA methyl esters were separated on fused-silica capillary column (CP-Sil 88; Chrompack, Middelburg, The Netherlands). An HPLC system equipped with three silver-impregnated silica columns (ChromSpher 5 Lipids, 250 × 4.6 mm, 5-µm particle size; Chromoptic, F-91971 Courtaboeuf) was used to separate methyl esters of CLA. Milk FA profiles, as described previously (Bougouin *et al.*, 2019) and summarised in online Supplementary Table 2.

Rumen fluid samples were collected by oral-stomach tubing. The system composed of a 300-cm tube (1.9-cm diameter) and a manual pumping system (Ammerlaan, Loue, France) was modified to have a collecting flask (1 l Duran borosilicate glass bottle) between the tube and the pump. Samples were taken on day 18 before the morning feeding (08.30h). The first 100–200 ml of rumen liquid collected were removed to avoid saliva contamination. The flask was emptied and replaced for the collection of approximately 500 ml of rumen fluid that were filtered through one layer of a polyester monofilament fabric (250 µm mesh aperture) and immediately 1 ml was aliquoted in 2-ml screw-cap tubes, centrifuged at 14 000 ***g*** for 10 min at 4°C and the pellet stored at −80°C. Faeces were collected manually the same day at the rectum, approximately 5 mg were aliquoted in 2-ml screw-cap tubes and stored at −80°C.

Microbial community analysis

Microbial DNA was extracted, both for rumen and faecal samples, following the protocol of Yu and Morrison, 2004. Briefly, the pellet of rumen fluid or whole faecal contents were thawed by adding 1 ml of lysis buffer (50 mM Tris-HCl pH8, 50 mM EDTA, 4% SDS, 500 mM NaCl), followed by a bead beating step at 5600 rpm for 3 × 60 sec with 5 s pause between cycles in a Precellys 24 machine (Bertin Instruments, Ozyme, Montigny-le-Bretonneux, France). Following the cell lysis step, DNA isolation and purification was performed according to QIAmp DNA Stool Mini Kit (QIAGEN FRANCE S.A.S). Genomic DNA (gDNA) was quantified on a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and integrity checked on a FlashGel System (Lonza, Rockland, Inc, France). Rumen and faecal gDNA was submitted to the Roy J. Carver Biotechnology Center (Urbana, IL61801, USA) for DNA library preparation using Fluidigm amplification and sequenced on an Illumina MiSeq platform using a MiSeq 600-cycle sequencing kit version 3. Selected primers for bacterial 16S rRNA were 515F (5′-GTGTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Hagey *et al.*, 2019) amplifying a fragment of 292 bp.

Sequencing data processing and multivariate analysis

Sequencing data are available on the NCBI Sequence Read Archive as BioProject ID PRJNA554264. Reads were merged

https://doi.org/10.1017/S0022029922000498 Published online by Cambridge University Press

using the make.contig function from mothur (Schloss *et al*., 2009) and resulting sequences were subjected to a quality filter (mean phred score ≥25, length ≥250, maximum 5 primer mismatches, <8 homopolymers). Trimmed reads were checked for chimaera using the chimera.uchime option in mothur. After chimaera removal, sequences were aligned using Pynast (Caporaso *et al*., 2010*a*). Operational taxonomical units (OTUs) were clustered at 99% of similarity using QIIME (Caporaso *et al*., 2010*b*), and taxonomy was assigned against the GreenGenes 13.8 database for Bacteria. Online Supplementary Table 3 summarises data analysis metrics. To check whether sequencing depth was adequate to characterise gut bacterial communities, Good's coverage was calculated using R package QsRutils. Coverage analysis showed that, on average, 90.8% and 78% of the OTUs, respectively, in the rumen and faecal contents were sampled more than once (online Supplementary Table 3). After rarefying to 2049 (rumen) and 6408 (faeces) reads to normalise for differential sequencing depths, coverage dropped to 78 and 59%, respectively. Alpha diversity was further analysed by computing diversity indices of Richness (number of species present), Shannon (how many different species are present), Simpson (how many different species are present, but giving more weight to dominant species), and Evenness (derived from the Shannon index, but refers to how even (close) in numbers the species in a given environment are) using the vegan (Oskansen *et al*., 2016) package. Finally, the Kruskal Wallis test was used to compare the species richness and alpha-diversity measurements between the treatments.

Taxonomic tables summarise cumulative abundance per taxonomic group (from the phylum to the genus). Values were log-transformed, and the non-parametric Kruskal-Wallis with Bonferroni correction test was used to check for differences in the relative abundance of taxa due to diet.

To visualise microbial trends associated with dietary treatments and identify microbial signatures, we performed UpSet plots using UpSetR in R (Conway *et al*., 2017). Further, the sparse Supervised Analysis and Selection of Discriminative OTUs (sPLS-DA) analysis as implemented in the mixomics R package (Rohart *et al*., 2017) was used. OTU tables were pre-processed by keeping only OTUs with at least 3 reads per sample, which were further TSS (Total Sum Scaling) transformed after adding pseudo counts. In detail, the discriminative features were selected from the rarified OTU tables. PLS-DA performance evaluation retained 3 components for both rumen and faecal data. The sPLS model was tuned to choose the distance giving lower error rates, and the final models used maximum distance, 3 components and 50 OTUs selected on each component. Selected OTUs on all retained components (artificial variable built from the linear combination of the observed variables) were clustered using the unweighted pair group method with arithmetic mean (UPGMA) algorithm and visualised using clustered image map built with the *cim* function from the mixomics package. Discriminant OTUs were visualised using *plotLoadings* function with the arguments contrib = 'max' and method = 'mean'. To investigate the links between microbiota and milk fatty acid profiles measured previously (Bougouin *et al*., 2019), we performed N-integration with sparse Discriminant Analysis on rumen and faecal bacteria OTU tables and milk FA concentrations. The aim was to select a subset of OTUs and milk FA that are highly positively or negatively correlated across the cows fed the four experimental diets. This analysis, integrated into the mixOmics package, is completely unsupervised: no prior knowledge about the samples groups is included. Circus plots visualised associations with a Pearson correlation coefficient higher than 0.9.

Results

Diet-related effects on bacterial community structure in rumen and faeces

Diversity indices for bacterial communities in the rumen and faeces were not affected by dietary treatment, except for Richness than was lower in the rumen of starch-fed cows and numerically (non-significanlty) higher in their faecal contents (online Supplementary Fig. S1). In faecal samples, variability (values dispersion) for Shannon, Simpson and Evenness in contents from cows supplemented with Ca salts of palm oil was low compared to the other dietary treatments (online Supplementary Fig. S1).

In rumen samples, sequences grouped in 18 phyla, 34 classes, 58 orders, 80 families, 148 genera and 172 species. The Bacteroidetes, Firmicutes and Proteobacteria were the most abundant phyla whatever the diet. At the Phylum level, Proteobacteria were numerically more abundant in lipid supplemented diets compared to high starch diet, but this difference was only significant with extruded sunflower supplementation (data not shown). At the order level, supplementation with extruded sunflower seeds increased the relative abundance of Aeromonadales ($P < 0.05$) and decreased ($P < 0.05$) the relative abundance of Erysipelotrichales (online Supplementary Table 4a). The relative abundance of unclassified Gammaproteobacteria-related sequences also significantly decreased with lipid-supplemented diets. At the genus level (Supplementary Table 4b), only two taxa were significantly affected by diet: *Sharpea* and unclassified *Ruminococcaceae,* whose relative abundances were lower and higher with extruded rapeseed compared to Ca salts palm oil and high starch diet.

An *upSetR* analysis was performed to identify core (present in all cows, independently of diet) and diet-specific OTUs in taxonomic datasets (Fig. 1). There were 78 core OTUs, and most of them affiliated to *Prevotella* (39%), unclassified *Succinivibrionaceae* (14%), *Succinivibrio* (10%) and *Fibrobacter* (10%). It should be noted that 86 OTUs were restricted to cows receiving the starch diet. Among those, some OTUs were classified as *Butyrivibrio* (10%), *Megasphaera* (11%), *Selenomonas bovis* (4%), *Shuttleworthia* (4%), *RFN20* (a rumen specific genus from the *Tenericutes* phylum) (4%) and these strains were not retrieved in rumen contents of cows fed any of the lipid-supplemented diets. Likewise, *Anaeroplasma*, *Pseudomonas thermotolerans*, and *RF39* related OTUs were specific to the rumen contents of cows supplemented with Ca salts of palm oil. This lipid-supplemented diet had the lowest number of non shared OTUs, twenty, compared to the diets supplemented with extruded sunflower seeds (47 specific OTUs) and/or extruded rapeseeds (45 specific OTUs). The OTUs affiliated as unclassified YS2 were only found in the contents of cows receiving extruded-sunflower seeds diets, while some Fibrobacter OTUs were specific to the diet with rapeseeds.

In faecal samples, we detected 15 phyla, 31 classes, 49 orders, 73 families, 147 genera and 181 species. Clostridiales dominated the faecal microbial community independently of the diet (online Supplementary Table 5). The only notable effect of the diet was an increase in the relative abundance of Fibrobacterales in the faeces of cows fed Ca salts of palm oil or extruded sunflower seeds (online Supplementary Table 5).

We used sPLS-DA to highlight diet-related changes in bacterial community structure and select bacterial OTUs in the rumen and faecal contents that discriminate the four dietary treatments. Figure 2a displays the clustered image map of the selected discriminant OTUs. Bacterial communities of cows supplemented

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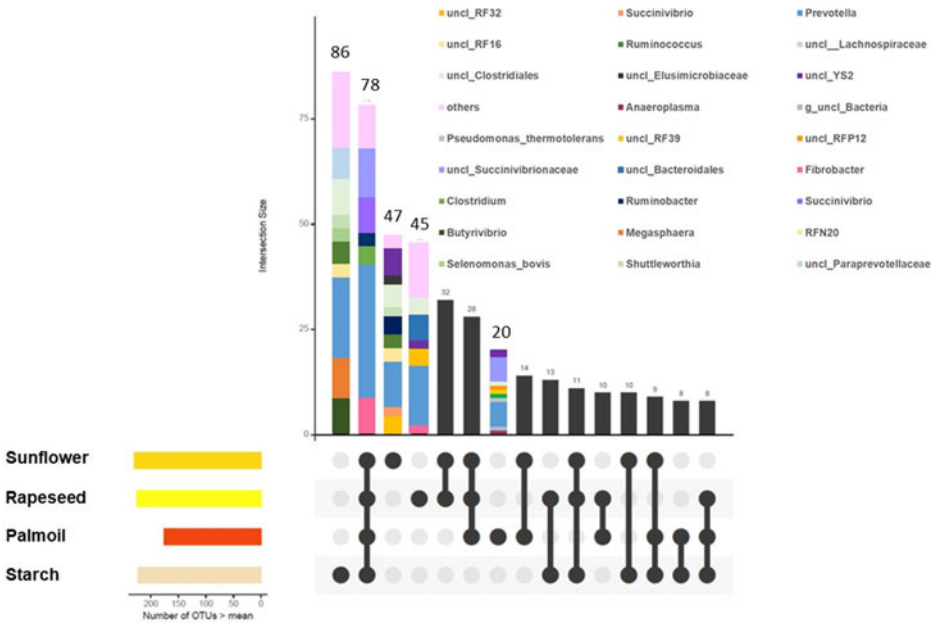

Fig. 1. An UpSetR plot of OTUs across rumen contents of cows fed Starch (high starch diet), Palmoil (rumen-protected SFA from Ca salts of palm oil), Rapeseed diet (rich in MUFA from extruded rapeseeds) or Sunflower diet (rich in PUFA from extruded sunflower seeds). Metadata for each diet is plotted on the left of the set size bars. Dark circles indicate samples with containing accessions and their counts are showed by the figures on the top of the charts. Connecting bars indicate overlap between multiple samples.

with Ca salts of palm oil and extruded sunflower seeds grouped apart from extruded rapeseed-supplemented and high-starch diets. The OTUs driving these differences were identified in the contribution plots of Fig. 2b. The separation of the high-starch diet on the first component is mainly due to Clostridiales-related genera, essentially *Butyrivibrio*. Bacteroidales-related taxa, mostly *Prevotella,* characterised the diet with rapeseeds on component 2. *Prevotella* and YS2-related OTUs were key variables driving the segregation of diets supplemented with Ca salts of palm oil or extruded sunflower seeds on component 3.

Similarly, in faecal contents, the clustered image map (Fig. 3a) highlighted diet-dependent clusters of faecal bacterial communities. The top 50 discriminating OTUs separated diets supplemented with Ca salts of palm oil or extruded rapeseeds from the starch and sunflower seed- supplemented diets. As revealed by the loading plots (Fig. 3b), most of these OTUs were affiliated with the Clostridiales order, particularly the *Ruminococcaceae*.

Establishing links between the rumen and faecal bacteria and milk fatty acid profiles

We explored the connection between the rumen and faecal bacterial community structure and milk FA profiles by extracting correlated datasets. The heatmap in Fig. 4 shows correlations (coefficient value ≥0.7) between rumen OTUs and FA concentrations in milk. Most linoleic acid isomers clustered together and positively correlated with taxa affiliated with the *YS2* family. A second cluster grouped the odd and branched-chain FA ($C_{15}:0$, $C_{17}:0$, $C_{13}:0$ and their isomers), which positively correlated with *Butyrivibrio*, *Megasphaera* and also *Prevotella* strains.

Further, we used the dimension reduction technique implemented in the Diablo method to select correlated and discriminatory variables from the three datasets (rumen and faecal OTU tables and milk FA profiles). The circos plot in Fig. 5 depicts the strongest correlation (coefficient value ≥0.9) between the different features of component 1. The heptadecanoic acid positively correlated with OTUs classified as *Prevotella*, *Butyrivibrio, Desulfovibrio, Bulleidia* and unclassified OTUs affiliated to *Veillonellacaea, Gammaproteobacteria, Lanchnospiraceae, Clostridiales* and *Bacteroidales* from rumen contents. The stearic acid correlated strongly with ruminal *Prevotella*, *Bulleidia* and unclassified *Veillonellaceae, Gammaproteobacteria, Lanchnospiraceae,* and *Bacteroidales*. Both FA and rumen OTUs presented a strong correlation with an unknown *Ruminococcaceae* from faecal contents.

Discussion

The study was conducted with dairy cows fed diets containing 40% concentrate rich either in starch or in SFA (Ca salts of palm oil), MUFA (extruded rapeseeds) or PUFA (extruded sunflower seeds). These diets promoted significant differences in milk FA profiles (Bougouin *et al.*, 2019). In the present work, we explored microbial drivers of observed changes in milk FA profiles. We focused on bacteria, as these procaryotes are mostly involved in rumen FA metabolism (Ferlay *et al.*, 2017). We adopted a high throughput sequencing approach followed by sparse partial least squares analysis to explore diet-driven changes in the rumen and faecal bacteria and their link with milk FA profiles. Diet is crucial in shaping rumen microbiota and, as a consequence, the host's phenotype (Henderson *et al.*, 2015; Huws *et al.*, 2018; Furman *et al.*, 2020). Our study showed that diet influenced bacterial community structure both in the rumen and faeces, but we did not uncover potential microbial biomarkers in the faeces that could act as proxies of community changes in the rumen.

In line with the literature (Huws *et al.*, 2018), Bacteroidetes, Firmicutes, and Proteobacteria were the principal phyla in the rumen ecosystem, while Firmicutes (mainly Clostridiales) dominated in faeces. In the rumen, one-third of all detected OTUs were shared between cows fed different diets. This is not surprising as a global assessment showed that core bacterial groups represented almost 90% of sequencing data (Henderson *et al.*, 2015). The diet did not influence diversity indices of Shannon, Simpson and Evenness of rumen and faecal contents, but Richness was lower in the rumen and numerically (non-significantly) higher in the faeces of cows receiving the high-starch diet. The starch content in the high-starch diet, provided

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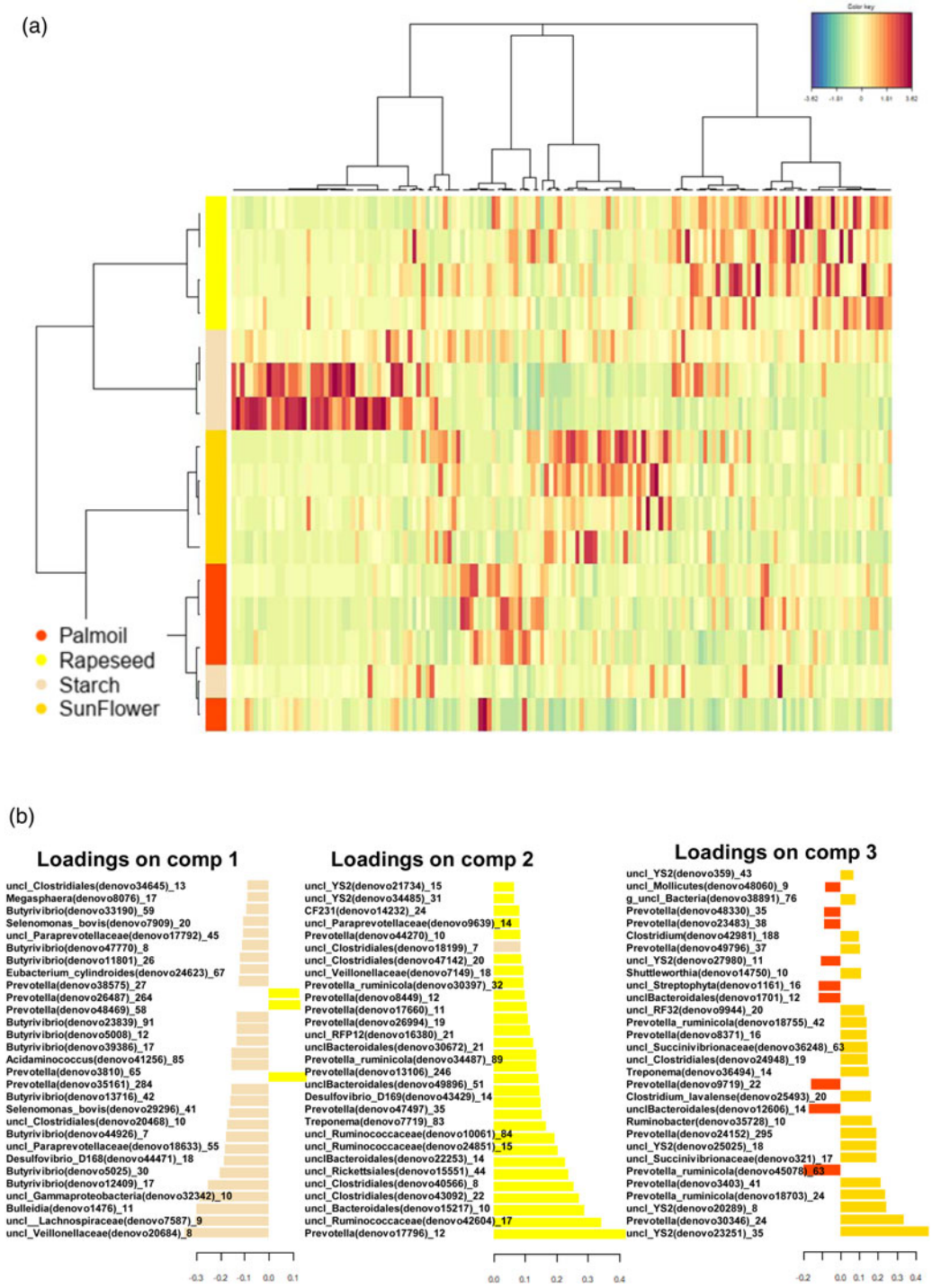

Fig. 2. Diet driven changes in bacterial community structure of the rumen in cows fed Starch (high starch diet), Palmoil (rumen-protected SFA from Ca salts of palm oil), Rapeseed diet (rich in MUFA from extruded rapeseeds) or Sunflower diet (rich in PUFA from extruded sunflower seeds). (a) Heatmap of selected OTUs on the three components using max.dist. Clustering of rows and columns was performed using Pearson's correlation. Red colour indicates a strong positive correlation and blue, strong negative correlation. (b) Loadings of discriminant OTUs identified on each component.

by cereals was almost 23% (DM basis), while it represented 18% in the lipid supplemented diets. We could speculate that the readily degradable starch from cereals decreased rumen pH after feeding, which could have a detrimental effect on some rumen bacteria (Russell and Dombrowski, 1980). Moreover, the readily fermentable substrate could also reduce ruminal degradation of plant cell walls and increase the amount of organic matter arriving in the hindgut, thus stimulating caecal microbes' growth and inducing changes in the bacterial communities that could be reflected in the faeces.

Even though species richness was lower in rumen samples from cows fed the high-starch diet, the number of OTUs non-shared with the lipid-supplemented diets was high. Several concurrent factors may be at play such as higher amount of

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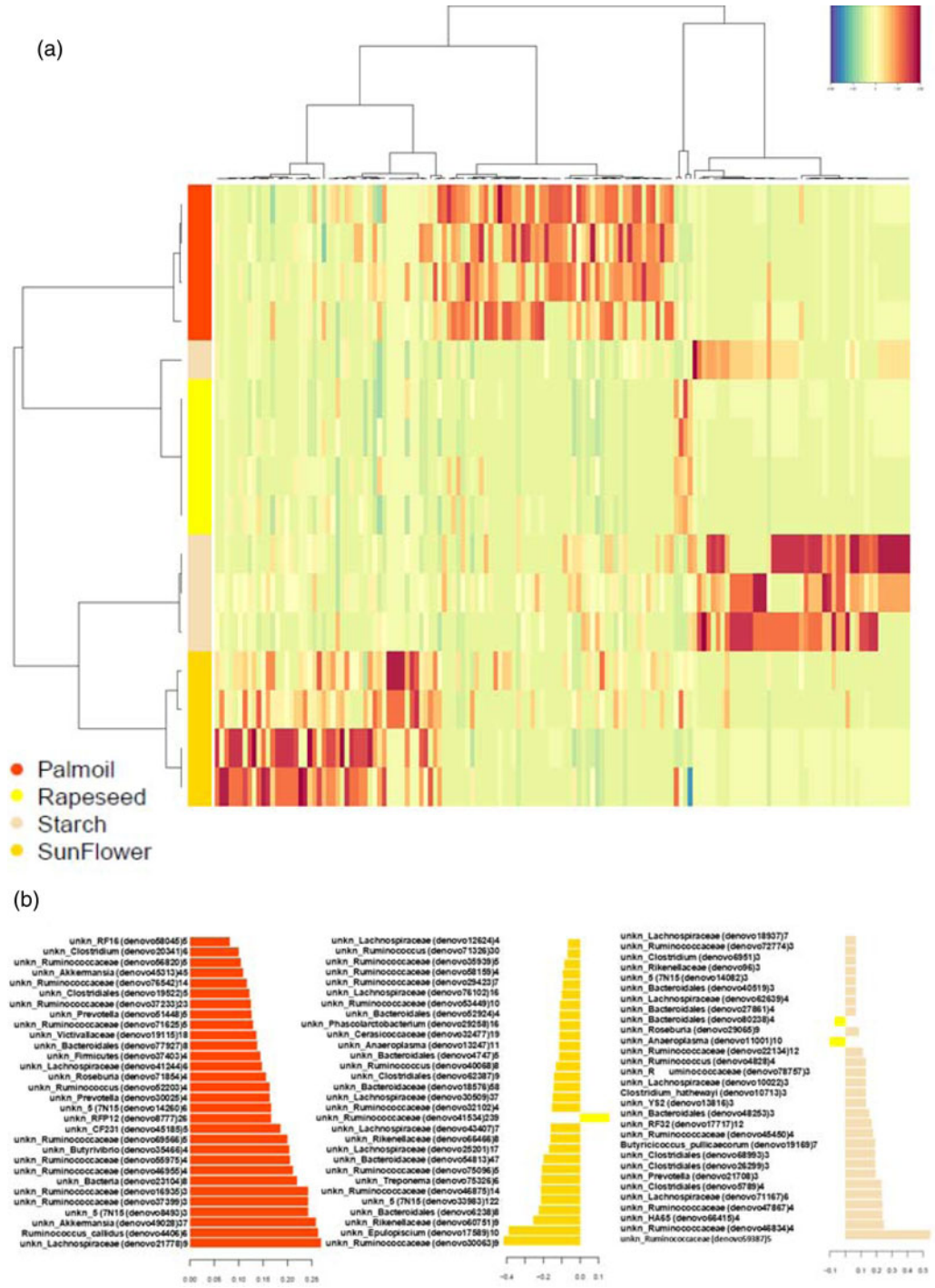

Fig. 3. Diet-driven changes in bacterial community structure of the faeces in cows fed Starch (high starch diet), Palmoil (rumen-protected SFA from Ca salts of palm oil), Rapeseed diet (rich in MUFA from extruded rapeseeds) or Sunflower diet (rich in PUFA from extruded sunflower seeds). (a) Heatmap of selected OTUs on the three components using max.dist. Clustering of rows and columns was performed using Pearson's correlation. Red colour indicates a strong positive correlation and blue, strong negative correlation. (b) Loadings of discriminant OTUs identified on each component.

fermentable substrate present in the diet that favours the growth of amylolytic and other starch-digesting bacterial species, while the drop in pH after feeding could be detrimental to others. In addition, supplementations with sunflower (Zened *et al.*, 2013), linseed (Popova *et al.*, 2019) or soybean and linseed (Yang *et al.*, 2009) have been shown to have an inhibitory effect mostly on cellulolytic microbes and, in particular, *Butyrivibrio* species. These findings are consistent with discriminant OTUs identified for the starch diet in our study, most of them classified as *Butyrivibrio*. *Butyrivibrio* species are involved in fibre breakdown, and many strains have a proteolytic activity or are also involved in the hydrolysis of lipids (Ferlay *et al.*, 2017). However, studies with a representative *Butyrivibrio* sp. (*B. fibrosolvens* JW11) showed that biohydrogenation is instead a detoxification process and not a way of obtaining energy for growth (Maia *et al.*, 2010). Reduced abundance, if not absence, of *Butyrivibrio*-related strains

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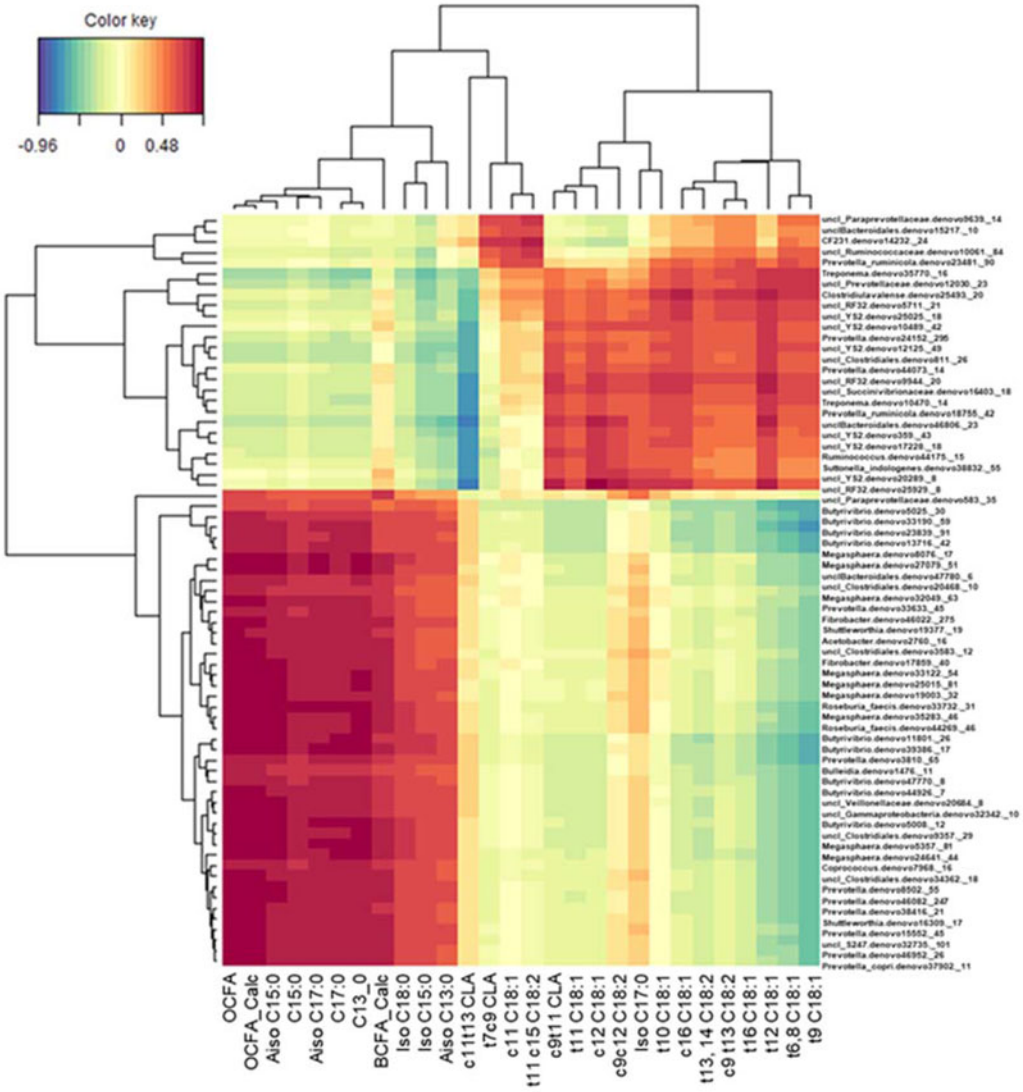

Fig. 4. Clustered image map showing correlations ($P > 0.7$) between discriminants OTUs from rumen contents and milk fatty acids concentrations from cows fed Starch (high starch diet), Palmoil (rumen-protected SFA from Ca salts of palm oil), rapeseed (rich in MUFA from extruded rapeseeds) or Sunflower diet (rich in PUFA from extruded sunflower seeds).

in rumen contents of lipid-supplemented cows could be explained by the bacteriostatic effects of unsaturated FA (Maia *et al*., 2010).

Regarding supplementation with Ca salts of palm oil, they should bypass degradation in the rumen and dissociate in the acid conditions of the abomasum, ready to be absorbed by the animal and enrich its products. However, against expectations, when cows were supplemented with Ca salts of palm oil, milk fat content decreased by 10% (Bougouin *et al*., 2019), and shifts in bacterial community were similar to what we observed with the unprotected sunflower seeds and rapeseeds. Consequently, it cannot be excluded that Ca salts of FA from palm oil were dissociated in the rumen.

Butyrivibrio biohydrogenation activity in the rumen consists of the isomerisation of linoleic acid to rumenic, vaccenic and finally stearic acids (Kepler *et al*., 1966). These latter FA are absorbed in the intestine and transported by the circulatory system to the mammary gland (Bernard *et al*., 2018). Our study showed no significant difference in the concentration of vaccenic and stearic acids in milk between cows fed starch, Ca salts of palm oil, extruded rapeseeds or sunflower seeds (Bougouin *et al*., 2019). However, in agreement with the higher *Butyrivibrio* abundance in the rumen of starch-fed cows, the concentration of rumenic acid in the milk with the starch diet was higher (significantly or numerically) than lipid-supplemented diets. *Megasphaera* strains are also recognised for their biohydrogenation activity in the rumen. Previous studies reported increased *Megasphaera* relative abundance on milk-fat depressing diets, and this taxon positively correlated with FA isomers with double bond *trans*10 (Pitta *et al*., 2019). *Megasphaera elsdenii* YJ-4 was shown to produce *trans10 cis12* CLA (Kim *et al*., 2002). In our study, *Megasphaera* was primarily found in the rumen with starch diets, but an increase in the concentration of *trans10 cis12* CLA in milk was observed with diets supplemented with extruded sunflower and Ca salts of palm oil.

Along with *Butyrivibrio* and *Megasphaera, Selemonas bovis, Shuttleworthia* or *RFN20* affiliated OTUs were distinctive features of the microbiota of cows fed starch diet. These rumen strains strongly correlated with OBCFA, $C_{15}:0$, and $C_{17}:0$, all having a

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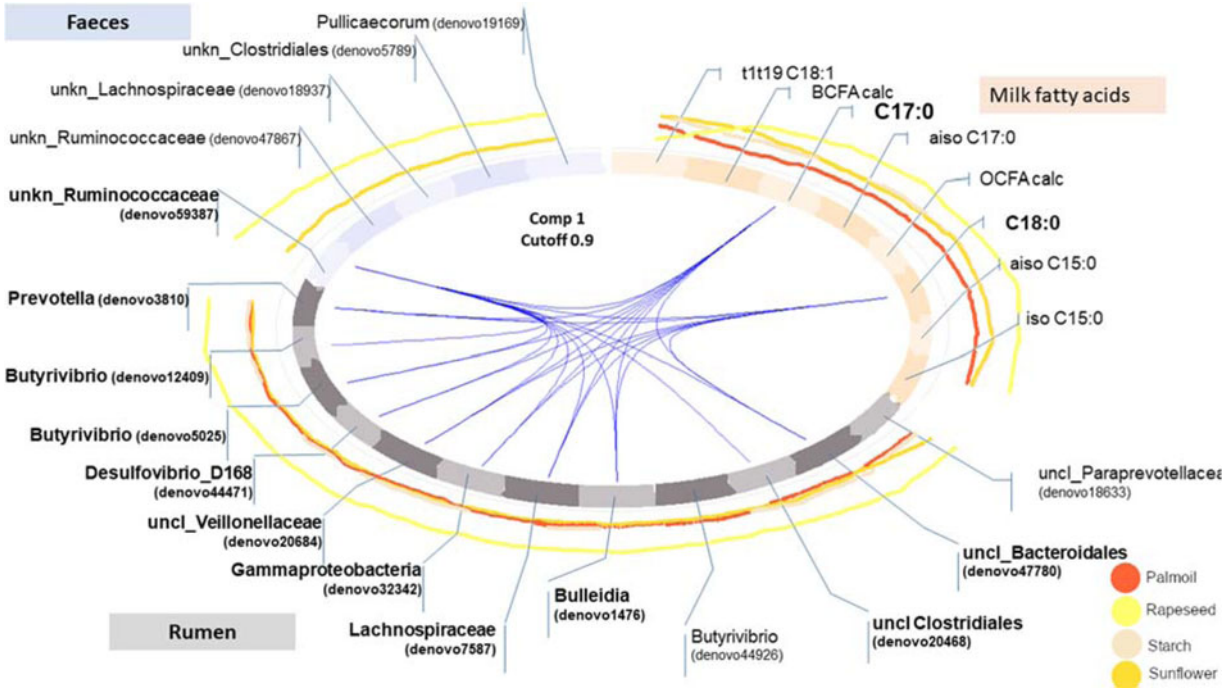

Fig. 5. The Circos plot showing correlation analysis of milk fatty acids profiles with microbial datasets. The positive correlations between selected features are denoted as blue lines; negative correlations were not depicted at the correlation cutoff of 0.9. Sparse partial least-squares discriminant analysis (sPLS-DA) was used to identify the first component based on milk fatty acids concentrations and OTU relative abundance in the rumen and faecal contents. The most discriminative features that were selected by the model from milk fatty acids (orange-rose) and OTU abundance in the rumen (grey) and faecal (blue) are shown; the outermost lines represent the feature concentration or relative abundance in samples from cows fed Starch (pale yellow), Palmoil (red), Rapeseed (yellow), Sunflower (orange) diets.

higher concentration in the milk of cows receiving the starch diet (Bougouin *et al.*, 2019). Although linear odd-chain FA of milk are de novo synthesised in the mammary gland, they primarily derive from rumen bacteria. Vlaeminck *et al.* (2006) suggested that diet-induced changes in milk OBCFA are mediated by changes in the structure of the rumen microbiota. They also summarised the OBCFA profile of predominant bacteria, and *Butyrivibrio, Selemonas ruminantium* and *Megasphaera elsdenii* were enriched in $C_{13}:0$, $C_{15}:0$ and $C_{17}:0$, as well as *iso* and *anteiso* $C_{15}:0$ for *Butyrivibrio.* As for *Shuttleworthia,* this genus belongs to the *Ruminococcaceae* family having members with a high content of odd-chain *iso*-FA. As previously suggested, milk OBCFA contents reflect rumen microbial community structure (Vlaeminck *et al.*, 2006) and, in particular, bacteria quitting the rumen. These findings suggest that milk FA profiles are influenced by both bacterial composition and activity in the rumen.

Moreover, OTUs discriminating rapeseeds, sunflower seeds and Ca salts of palm oil supplemented diets belonged to unclassified Bacteroidales or *Prevotella,* metabolically flexible taxa, but also YS2/Cyanobacteria. A recent genomic study classified *YS2* into the new class Melainabacteria. Their predicted metabolic profile suggests that these are obligate anaerobic fermenters that can use a broad set of carbohydrates, but also organic, amino and FA to yield hydrogen, acetate, formate or butyrate (Di Rienzi *et al.*, 2013). *Prevotella* is part of the rumen microbiota core (Henderson *et al.*, 2015) and *YS2* is present in low proportions (Neves *et al.*, 2017). However, given the toxic effect of lipids on other microbes, as well as the presence of new substrates (FA), low abundant members of these nutritionally versatile phyla might have found a way to expand their niche. OTUs classified as *Prevotella* and *YS2* presented a strong positive correlation with $C_{18}:1$ and $C_{18}:2$ isomers with concentrations higher in all or at least one of the lipid-supplemented diets, compared to starch diet (Bougouin *et al.*, 2019). Analysis performed here is of a co-occurrence type, based primarily on the relative abundance of detected taxa and FA concentrations in milk. Discussing microbial activity based on this analysis will be too speculative but we can presume that these bacterial populations could be implicated in the rumen biohydrogenation process. Measuring FA profiles of rumen contents coupled with analysis of microbial activity and strain isolation should be the next step to have a better insight on the role of these bacteria on FA metabolism and milk fat composition.

Additionally, we examined faecal microbiota not only because the caecal fermentation may supply up to 9% of the gross energy available to dairy cows, but also because faecal samples could be used as a proxy to monitor rumen microbiota. We adopted a method of discriminant analysis, allowing us to highlight diet-induced changes in faecal microbiota. Our results showed that faecal bacteria communities of starch and sunflower seed-fed cows clustered separately from Ca salts of palm and rapeseed-supplemented cows. Selected OTUs from the multivariate model, were mostly classified as *Ruminococcaceae* on each of the three components retained in the analysis. Revealed changes in faecal bacterial community structure could be explained by the diet-induced shifts of the rumen microbiota, as well as modification in the amount and/or nature of substrate reaching the caecum. The final step integrating discriminant OTUs from faecal and rumen contents with FA concentration in milk demonstrated strong correlation of $C_{15}:0$ and $C_{17}:0$ with 10 rumen taxa (essentially *Butyrivibrio*, other *Lachnospiraceae* or Clostridiales), but only one faecal strain (unknown *Ruminococcaceae*). This could

https://doi.org/10.1017/S0022029922000498 Published online by Cambridge University Press

be due to the higher individual related variability in faecal microbiota. A recent study from a large cohort of dairy cows ($n = 150$) demonstrated that though 95% of the faecal samples shared 27 genera, there was only one core OTU (Hagey *et al.*, 2019). Thus, a better characterisation of individual bacterial strains is essential for an enhanced understanding of gut microbial ecosystems.

Conclusions

In this work we explored the correlation between diet-induced changes in digestive bacterial communities and milk FA profiles in dairy cows. Our results show that lipid-supplemented diets, compared to high-starch diet, decreased concentrations of $C_{15}:0$ and $C_{17}:0$ and increased content of *trans* and *cis* isomers of $C_{18}:1$ and $C_{18}:2$ in milk by affecting bacterial strains performing PUFA biohydrogenation processes in the rumen. Moreover, lipid supplementation increased the proportion of bacteria with versatile metabolism, namely *Prevotella* and *YS2*. The effects of these bacterial populations on FA metabolism in the rumen and on FA composition in milk need to be further investigated. In this study we did not demonstate the potential of faecal bacterial to depict rumen changes associated to milk FA profiles. However, we highlighted diet induced shifts in faecal bacterial structure.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000498.

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