Animal 16 (2022) 100520

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Exploring the relationship between bacterial genera and lipid metabolism in bovine rumen

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ARTICLE INFO

Article history:
Received 15 November 2021
Revised 15 March 2022
Accepted 18 March 2022
Available online 22 April 2022

Keywords:
Biohydrogenation
Canonical correlation analysis
Dimethylacetals
Fatty acid
Rumen microbiota

ABSTRACT

The rumen is characterised by a complex microbial ecosystem, which is particularly active in lipid metabolism. Several studies demonstrated a role of diet and breed on bacterial community profile, with the effect on metabolic pathways. Despite the knowledge achieved on metabolism and the bacterial profile, little is known about the relationship between individual bacteria and metabolic pathways. Therefore, a multivariate approach was used to search for possible relationships between bacteria and products of several pathways. The correlation between rumen bacterial community composition and rumen lipid metabolism was assessed in 40 beef steers (20 Maremmana and 20 Aubrac) reared with the same system and fed the same diet. A canonical discriminant analysis combined with a canonical correlation analysis (**CCA**) was performed to explore this correlation. The variables showing a Pearson correlation higher than 0.6 as absolute value and significant were retained for CCA considering the relationship of bacterial composition with several metabolic pathways. The results indicated that some bacterial genera could have significant impacts on the presence of several fatty acids. However, the relationship between genera and fatty acid changes according to the breed, demonstrating that the metabolic pathways change according to the host genetic background, related to breed evolution, although there is also an intra-breed genetic background which should not be ignored. In Maremmana, *Succiniclasticum* and Rikenellaceae_RC9_gut_group showed a high positive correlation with dimethylacetals (**DMAs**) $DMA_{C13:0}$, $DMA_{C14:0}$, $DMA_{C14:0iso}$, $DMA_{C15:0}$, $DMA_{C15:0iso}$, and $DMA_{C18:0}$. Prevotellaceae_UCG-003 correlates with C18:3c9c12c15 and C18:1t11, while *Fibrobacter* and *Succiniclasticum* correlate with C18:2c9t11 and Lachnospiraceae_NK3A20_group correlates with C18:1c12. Prevotellaceae_UCG-003, Ruminococcaceae_UCG-010, and *Oribacterium* showed a positive correlation with C13:0iso, and C17:0. Conversely, in Aubrac, Treponema_2 and Rikenellaceae_RC9_gut_group correlated with $DMA_{C14:0iso}$, $DMA_{C16:0iso}$, $DMA_{C17:0iso}$, while Ruminococcaceae_UCG-010, Christensenellaceae_R-7_group and Ruminococcaceae_NK4A214_group correlated with $DMA_{C18:1t11}$, $DMA_{C14:0}$, $DMA_{C18:1c12}$. *Acetitomaculum* correlated with C18:2c9c12, C18:1c12, C18:1c13, C18:1t12 and Lachnospiraceae_NK3A20_group with C18:1t6-8 and C18:1t9. *Saccharofermentas*, Ruminococcaceae_UCG-010 and Rikenellaceae_RC9_gut_group correlated with C18:2c9t11 while, Prevotellaceae_UCG-001 and Ruminococcus_1 correlated with C14:0iso, C15:0, C15:0iso, C17:0. *Saccharofermentans*, Rikenellaceae_RC9_gut_group, Ruminococcaceae_NK4A214_group, and Ruminococcaceae_UCG-010 correlated with C13:1c12 and C16:0iso. These results lead to hypothesise a possible association between several metabolic pathways and one or a few bacterial genera. If these associations are confirmed by further investigations that verify the causality of a bacterial genus with a particular metabolic process, it will be possible to deepen the knowledge on the activity of the rumen population in lipid metabolism. This approach appears to be a promising tool for uncovering the correlation between bacterial genera and products of rumen lipid metabolism.

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https://doi.org/10.1016/j.animal.2022.100520
1751-7311/

Implications

The role of the rumen bacterial population is a critical point in the study of lipid metabolism, due to its implications on the animal wellness and the quality of products. However, the information available is limited due to the difficulty in obtaining reliable phenotypes and the complexity of modelling the ruminal environment. Therefore, a multivariate statistical approach was performed to simplify the complex system that characterises the rumen environment to provide a simpler interpretation that regulates the lipid metabolism of the rumen. We found a close relationship of some bacteria with certain products, suggesting their direct role in particular pathways.

Introduction

The rumen environment is characterised by a complex microbial ecosystem (Puniya et al., 2015; Knoell et al., 2016), consisting of bacteria, archaea, fungi, and ciliated protozoa (Mackie et al., 2000). The bacterial component is particularly active in rumen lipid metabolism, which can be summarised in three important steps: lipolysis, biohydrogenation and *de novo* synthesis of fatty acids (Alves et al., 2013). In the last years, microbial lipid metabolism has appealed particular interest, because different metabolites bypass the rumen accumulating in the different tissues and define the fatty acid profile of animal origin products (i.e., milk, meat) (Alves et al., 2013).

Dietary lipids, particularly triacylglycerols and the unesterified fatty acids produced by microbial lipolysis, have been shown to be firmly adsorbed onto the surface of rumen particulate matter by hydrophobic interactions, while they are absent in the liquid phase of digesta (Buccioni et al., 2012). Immediately after ingestion, dietary lipids (triglycerides, phospholipids and galactolipids) are hydrolysed in the rumen by microbial lipases, which release free fatty acids (Harfoot and Hazelwood, 1988; Buccioni et al., 2012). After lipolysis, the free polyunsaturated fatty acids (**PUFAs**) undergo a biohydrogenation process due to the activity of bacteria (Buccioni et al., 2012).

The biohydrogenation of dietary PUFAs is a metabolic process characteristic of rumen microbiota. Rumen microorganisms by biohydrogenation isomerise and hydrogenate dietary unsaturated fatty acids (as linoleic acid and α-linolenic acid) generating numerous isomeric unsaturated forms, commonly with trans double bonds, and saturated fatty acids. Bacteria and protozoa are also able to synthesise and incorporate long-chain fatty acids, mainly odd chain fatty acids (**OCFAs**) and branched-chain fatty acids (**BCFAs**), that are synthesised from propionate and/or branched-chain volatile fatty acids derived from branched-chain amino acids (Vlaeminck et al., 2006). Moreover, bacteria are also responsible for a high proportion of plasmalogen lipids, containing alk-1-enyl (vinyl) ether chains (herein named dimethylacetals, **DMAs**) (Miyagawa, 1982; Alves et al., 2013).

Belenguer et al (2010) found significant effects of dietary lipids on rumen bacteria community and the involvement of different genera in rumen lipid metabolism (Belenguer et al., 2010). A recent *in vivo* study (Daghio et al., 2021) on beef cattle showed a different rumen microbial profile between two bovine rustic breeds, with significant effects on lipid metabolism. Despite this, the technique adopted in that work did not allow to establish a direct relationship between individual bacteria and metabolic pathways, confirming the scarce information available about the role of specific genera in lipid metabolism *in vivo* (Boeckaert et al., 2008). The little information available in literature is mainly due to the synergistic interaction of rumen microorganisms that involves a bacterial consortium, with each group having a part in the synthesis and modification of rumen fluid fatty acids (Harfoot and Hazelwood, 1988). The high number of fatty acids and symbiotic bacteria involved in rumen lipid metabolism further complicates the interpretation of the phenomenon, due to the redundancy of information that each single variable provides and their close interaction in metabolic processes. Moreover, despite several studies examine the fatty acid metabolism, there is a lack of *in vivo* studies about the relationship between lipid metabolism products and bacterial strains (Shingfield et al., 2008; Kairenius et al., 2018). Multivariate data analysis techniques based on discriminant analysis and CCA could be adapted to obtain reliable results to better understand the effects of different bacterial genera on fatty acids directly involved in rumen lipid metabolism. In particular, CCA is a common feature extraction method in multivariate statistical analysis, which offers a useful system of measuring the linear relationship between two multidimensional variables. For this reason, CCA could be utilised to discover the fatty acids potentially associated with the bacterial genera present in the rumen.

The aim of this study was to investigate the role of several bacterial genera in the rumen lipid metabolism, trying to reveal specific association with particular lipid products, using a multivariate approach to analyse data from an *in vivo* study (Daghio et al., 2021). For this purpose, two breeds, Maremmana and Aubrac, were considered to evaluate the rumen metabolism, in relation to the interaction between the microbial population and the ruminal environment of the host. For this reason, an autochthonous breed (Maremmana, typical of the area where the test took place – central Italy) and one coming from France (Aubrac) were considered.

Material and methods

Experimental design and samples

The work was based on data obtained in an *in vivo* trial that was conducted by Daghio et al. (2021). Briefly, 40 steers of Maremmana and Aubrac breed (n = 20 each; average BW of 250 kg and 4.5 months old) were allocated into two experimental groups as follows: 10 Aubrac and 10 Maremmana were fed in a feedlot (2 500 m^2), whereas 10 Aubrac and 10 Maremmana were fed in a grazing (10 ha) system. The steers in the feedlot were fed grass hay ad libitum and concentrated feed (1 kg/100 kg of live weight per head and per day), whereas grazing animals received grass hay ad libitum in addition to the fresh forage available on the pasture that consisted of 62% grass, 17.5% legumes (mainly white clover), and 20.5% other species (Supplementary Table S1). The same kind of concentrate feed used for steers in the feedlot was administered to grazing steers when grass availability was limited (the amount of concentrate was decided monthly on the basis of the individual average daily weight gain and was not greater than 1 kg/100 kg of live weight per head). All the animals had free access to water. Rumen liquor samples were collected from freshly slaughtered steers and filtered on sterile cheese cloth to obtain 200 mL of sample for chemical and microbial analysis (Daghio et al., 2021). Approximately 2 mL of rumen liquor was immediately stored at −80 °C for microbial DNA extraction. The rest of the samples were stored at −20 °C and then lyophilised (ScanVAC Coolsafe 55-4 lyophilizer, LaboGene ApS DK-3450 Allerød) for the analysis of lipid profile composition.

Analysis of rumen bacterial communities and lipid metabolism products

All the analyses were described in Daghio et al. (2021). Briefly, fatty acids and DMA profile were characterised by gas-chromatograph analysis using a GC2010 Shimadzu gas chro-

matograph (Shimadzu, Columbia, MD, USA). Data related to c9t11, t8c10 and t7c9 isomers of C18:2 concentration were approximate to C18:2 c9t11 only considering this isomer as the main present in rumen liquor (more than 90%; Bauman et al., 2001). In turn, the microbial community characterisation of rumen liquor was performed by the sequencing of bacterial 16S rRNA gene amplicons as described by Daghio et al. (2021). Briefly, Operational Taxonomic Units (97% similarity) were generated using USEARCH 8.1 (Edgar, 2010 and 2013) and the taxonomic classification (80% confidence) was performed at genus level against SILVA database v138 (Pruesse et al., 2007). The sequences are available at the National Centre for Biotechnology Information (NCBI) under BioProject number PRJNA682716. A detailed description of all the analyses performed is given in the Supplementary Material S1.

Statistical analysis

Data were analysed by using the SAS software (ver. 9.4, SAS Institute Inc., Cary, NC). All data were divided into three distinct datasets as follows: (i) rumen DMA composition (dataset A), (ii) rumen fatty acids involved in biohydrogenation (**BHFAs**) pathways (dataset B), and (iii) rumen OCFA and BCFA composition (dataset C). All datasets were arranged in a multivariate manner with animals in rows (40 steers) and, as columns, bacteria in all datasets, plus the DMAs in A, the BHFAs in B and the OCFAs and BCFAs in C. Data were submitted to a multivariate statistical procedure, the canonical discriminant analysis (**CDA**), to test if DMAs, BHFAs, OCFAs and BCFAs and bacterial genera were respectively able to discriminate the Maremmana breed from the Aubrac and to discriminate feedlot from grazing system. If d indicates the number of involved groups, CDA derives d − 1 equations, called canonical functions that are linear combinations of the original variables. These new variables are used to predict the group to which an object belongs. In the present research, being groups = 2, only one canonical function for each CDA was obtained. The structure of a canonical function is as follows:

$$\text{Canonicalfunction} = c_1X_1 + c_2X_2 + \cdots + c_nX_n$$

where c_i are the canonical coefficients (**CCs**) and X_i are the scores of the original variables (DMAs, BHFAs, OCFAs and BCFAs and genera). Canonical coefficients indicate the partial contribution of each variable in composing the canonical function. In consequence, the higher the absolute value of a CC, the higher the weight of the corresponding variable in composing the canonical function. The distance between groups was evaluated by using the Mahalanobis' distance, whereas the effective group separation was tested by using the corresponding Hotelling's T-square test (De Maesschalck et al., 2000). Then, for each dataset, to consider only the most representative variables, the Pearson's correlations between genera and DMAs, BHFAs and odd and branched-chain fatty acids (**OBCFAs**), respectively, were calculated, separately both for Maremmana and Aubrac. In the six datasets (A, B, C × Maremmana, Aubrac), only variables whose correlations were higher than 0.60 were retained.

The CCA was used to investigate relationships among genera and DMAs, BHFAs and OCFAs and BCFAs, respectively. Genera were considered as independent variables, whereas DMAs, BHFAs and OCFAs and BCFAs were considered as dependent variables. If n and p, with $n < p$, are the number of the two analysed groups of variables, CCA derives, in each group, n linear combinations of the original variables called canonical variables. The procedure evaluates the relative contribution of each original variable to the derived canonical variable in order to explain the nature of the relationship(s). Canonical variables are so formed that the first pair, one for each group, has the largest correlation of any linear combination of the original variables; the second pair has the largest correlation of the subsequent linear combination and so on. A large canonical correlation between two canonical variables, however, does not mean that there is a strong relationship between the two sets of original variables. Canonical correlation only maximises the correlation between the two canonical variables, but does not maximises the amount of variances accounting for one set of variables by the other set of variables. Therefore, when a canonical correlation was significant, the redundancy index was calculated just to determine how much of the variation in the dependent variables (DMAs, BHFAs and OCFAs and BCFAs) is accounted for by the independent variables (genera) (Sharma, 1996).

Results

Discriminant analysis

A CDA was performed for each of the rumen pathways of lipid metabolism (DMAs synthesis, biohydrogenation and OCFAs and BCFAs synthesis) and bacterial community profiles, in order to assess whether there were fatty acids or bacteria characterised by one breed rather than another. In all cases, the extracted canonical functions significantly discriminated the two breeds (*P*-value Hotelling's *t*-test < 0.0001) with a 100% of correct classification. The variables that best explained the discrimination (CC > 0.5 in absolute values) for each of the three pathways are shown in Table 1. In particular, the Maremmana breed was characterised by $DMA_{C16:0}$, $DMA_{C14:0}$, $DMA_{C15:0ante}$ and $DMA_{C17:0iso}$, whereas for Aubrac, the most important DMAs were $DMA_{C15:0iso}$ and $DMA_{C13:0}$. Also BHFAs were able to perfectly discriminate the two breeds. In this case, C18:1t9, C18:1t15, C18:1t10 and C18:2c9c12 were associated with Maremmana, while C18:0, C18:1t6-8, C18:3c9c12c15, C18:1t16, C18:1c11, C18:1t12, C18:2c9t11 and C18:1c13 were correlated with Aubrac. Finally, the OCFAs and BCFAs that better discriminate the two breeds were C16:0iso, C13:1c12 and C15:0iso for Maremmana and C15:0ante and C15:0 for Aubrac. A perfect discrimination was also observed considering bacterial genera as variables: Ruminococcaceae_UCG-010, Ruminococcus_2, Christensenellaceae_R-7_group, and Prevotellaceae_UCG-001 characterised Maremmana breed, while Prevotella_1, Ruminococcaceae_NK4A214_group, *Succiniclasticum*, *Oribacterium*, Treponema_2, Ruminococcus_1, and *Fibrobacter* are involved in Aubrac discrimination.

In the same way, the CDA carried out to verify whether it was possible to identify fatty acids or bacteria capable of discriminating between the two farming systems (feedlot and pasture) showed that was not possible to observe a clear discrimination between feedlot and grazing system (*P*-value Hotelling's *t*-test > 0.05) with a low level of correct classifications (<60%).

Canonical correlation analysis

Bacterial taxa and dimethylacetals

The Pearson's correlation analysis selected for the Maremmana breed involved the following variables: $DMA_{C13:0}$, $DMA_{C13:0iso}$, $DMA_{C14:0}$, $DMA_{C14:0iso}$, $DMA_{C15:0}$, $DMA_{C15:0iso}$, $DMA_{C17:0ante}$, $DMA_{C18:0}$ for DMA, and *Acetitomaculum*, Rikenellaceae_RC9_gut_group, Ruminococcaceae_NK4A214_group, Ruminococcaceae_UCG-014, Ruminococcus_2, *Succiniclasticum*, Candidatus_Saccharimonas, Lachnospiraceae_NK3A20_group for bacterial genera. Regarding the Aubrac breed, the variables selected were $DMA_{C13:0iso}$, $DMA_{C14:0}$, $DMA_{C14:0iso}$, $DMA_{C15:0ante}$, $DMA_{C16:1}$, $DMA_{C16:0iso}$, $DMA_{C17:0iso}$, $DMA_{C18:1c11}$, $DMA_{C18:1c12}$, $DMA_{C18:1t11}$ for DMA, and Christensenellaceae_R-7_group, Ruminococcaceae_NK4A214_group, Lachnospiraceae_NK3A20_group, Rikenellaceae_RC9_gut_group, Ruminococcaceae_UCG-010, Treponema_2 for bacterial genera. Fig. 1 presents a similarity

Table 1
Canonical coefficients (CCs) obtained in the canonical discriminant analysis to discriminate Maremmana from Aubrac using dimethylacetals (DMAs), fatty acids involved in biohydrogenation (BHFAs), odd linear and branched fatty acids (OCFAs and BCFAs) and genera.

DMA	CC	BHFA	CC	OCFA and BCFA	CC	Genera	CC
$DMA_{C15:0iso}$	2.41	C18:1t6-8	13.96	C15:0ante	0.77	Prevotella_1	1.63
$DMA_{C13:0}$	0.67	C18:0	1.56	C15:0	0.62	Ruminococcaceae_NK4A214_group	1.27
$DMA_{C17:1}$	0.39	C18:3c9c12c15	1.22	C13:0iso	0.43	*Succiniclasticum*	1.18
$DMA_{C17:0}$	0.36	C18:1t16	1.18	C17:0	0.32	*Oribacterium*	0.76
$DMA_{C18:1t11}$	0.33	C18:1c11	0.95	C17:0iso	0.12	Treponema_2	0.71
$DMA_{C18:1c9}$	0.26	C18:1t12	0.90	C14:0iso	−0.03	Ruminococcus_1	0.69
$DMA_{C18:0}$	0.09	C18:2c9t11	0.68	C13:0	−0.26	*Fibrobacter*	0.67
$DMA_{C16:1}$	0.09	C18:1c13	0.57	C17:0ante	−0.28	Rikenellaceae_RC9_gut_group	0.47
$DMA_{C13:0iso}$	0.07	C18:1t11	0.12	C15:0iso	−0.57	Candidatus_Saccharimonas	0.45
$DMA_{C14:0iso}$	−0.07	C18:1c12	−0.14	C13:1c12	−0.63	*Saccharofermentans*	0.38
$DMA_{C17:0ante}$	−0.13	C18:2c9c12	−0.78	C16:0iso	−0.98	Prevotellaceae_UCG-003	0.34
$DMA_{C18:1c12}$	−0.19	C18:1t10	−1.49			Lachnospiraceae_NK3A20_group	0.26
$DMA_{C15:0}$	−0.21	C18:1t15	−1.60			*Acetitomaculum*	0.15
$DMA_{C18:1c11}$	−0.22	C18:1t9	−12.82			Ruminococcaceae_UCG-014	0.02
$DMA_{C16:0}$	−0.25					Prevotellaceae_UCG-001	−0.54
$DMA_{C17:0iso}$	−0.54					Christensenellaceae_R-7_group	−0.99
$DMA_{C15:0ante}$	−0.59					Ruminococcus_2	−1.05
$DMA_{C14:0}$	−0.96					Ruminococcaceae_UCG-010	−1.32
$DMA_{C16:0}$	−1.35						

Abbreviations: DMAs = dimethylacetals; CCs = canonical coefficients; BHFAs = fatty acids involved in biohydrogenation; OCFAs = odd chain fatty acids; BCFAs = branched-chain fatty acids.

map of CCA, wherein raw data are shown in Supplementary Tables S2 and S3. One significant canonical variable was extracted in Maremmana breed, with a canonical correlation coefficient equal to 0.99. On the other hand, two canonical variables were extracted in Aubrac breed, with canonical correlation coefficients of 0.98 and 0.97 for the first and the second canonical variables, respectively. The relationships between the DMAs and bacterial genera are mapped in Fig. 1A and B for Maremmana and Aubrac, respectively. Coefficients in canonical variables with absolute values >0.5 are considered important, as reported in previous works (Bula et al., 2019; Quan et al., 2021). Thus, in Maremmana breed, *Succiniclasticum* and Rikenellaceae_RC9_gut_group showed a higher positive correlation with $DMA_{C13:0}$, $DMA_{C14:0}$, $DMA_{C14:0iso}$, $DMA_{C15:0}$, $DMA_{C15:0iso}$, $DMA_{C18:0}$, while *Acetitomaculum*, Ruminococcaceae_NK4A214_group, Ruminococcaceae_UCG-014, Ruminococcus_2, Lachnospiraceae_NK3A20 showed negative correlation with the same DMAs. Conversely, in Aubrac breed, we observed a high positive correlation of Treponema_2 and Rikenellaceae_RC9_gut_group with $DMA_{C14:0iso}$, $DMA_{C16:0iso}$, and $DMA_{C17:0iso}$, while Ruminococcaceae_UCG-010, Christensenellaceae_R-7_group and Ruminococcaceae_NK4A214_group were positively correlated with $DMA_{C18:1t11}$, $DMA_{C14:0}$, and $DMA_{C18:1c12}$.

For Maremmana, the raw variance of the DMA variables explained by the genera variables (redundancy analysis) was 49%, whereas for Aubrac, it was 56.5%.

Bacterial taxa and biohydrogenation intermediates

The variables used for the CCA in Maremmana breed were C18:1c12, C18:1t12, C18:1t16, C18:0, C18:3c9c12c15, C18:1t11, and C18:2c9t11 for BHFAs, and Candidatus_Saccharimonas, *Fibrobacter*, Lachnospiraceae_NK3A20_group, Prevotellaceae_UCG-003, Rikenellaceae_RC9_gut_group, and *Succiniclasticum* for bacteria. Regarding Aubrac breed, the variables selected were C18:1t12, C18:2c9c12, C18:1c12, C18:1c13, C18:1t6-8, C18:1t9, C18:1t15, C18:1c11, and C18:2c9t11 for BHFAs, and *Acetitomaculum*, Lachnospiraceae_NK3A20_group, *Oribacterium*, Rikenellaceae_RC9_gut_group, Ruminococcaceae_UCG-010 and *Saccharofermentas* for bacteria. Two significant canonical variables were extracted in Maremmana breed, with a canonical correlation coefficient ranging from 0.94 and 0.97 (Supplementary Table S4). Two canonical variables were extracted in Aubrac breed, with canonical correlation coefficients of 0.99 and 0.97 for the first and the second canonical variables, respectively (Supplementary Table S5). In Maremmana breed, Prevotellaceae_UCG-003 correlates with C18:3c9c12c15 and C18:1t11, *Fibrobacter* and *Succiniclasticum* correlate with C18:2c9t11, and Lachnospiraceae_NK3A20_group correlates with C18:1c12 (Fig. 2A).

Conversely, in Aubrac, a high positive correlation of *Acetitomaculum* with C18:2c9c12, C18:1c12, C18:1c13, C18:1t12 was observed, as well as for Lachnospiraceae_NK3A20_group with C18:1t6-8 and C18:1t9, and likewise for *Saccharofermentas*, Ruminococcaceae_UCG-010 and Rikenellaceae_RC9_gut_group with C18:2c9t11 (Fig. 2B). For Maremmana, the raw variance of the BHFAs variables explained by the genera variables (redundancy analysis) was 24%, whereas for Aubrac, it was 52%.

Bacterial taxa and odd and branched-chain fatty acids

The variables used for the CCA in Maremmana breed were C13:0iso, C15:0iso, C15:0, C16:0iso, C17:0iso, and C17:0ante for OCFAs and BCFAs, and *Acetitomaculum*, Candidatus_Saccharimonas, Christensenellaceae_R-7_group, Lachnospiraceae_NK3A20_group, *Oribacterium*, Prevotellaceae_UCG-003, Rikenellaceae_RC9_gut_group, Ruminococcaceae_NK4A214_group, and Ruminococcaceae_UCG-010, Ruminococcaceae_UCG-014 for bacterial genera. Regarding Aubrac breed, the variables selected were C13:0, C14:0iso, C13:1c12, C15:0iso, C15:0, C16:0iso, and C17:0 for OCFAs and BCFAs, and Prevotellaceae_UCG-001, Rikenellaceae_RC9_gut_group, Ruminococcaceae_NK4A214_group, Ruminococcaceae_UCG-010, Ruminococcus_1, *Saccharofermentans*, and Treponema_2 for bacterial genera. Fig. 3 presents a similarity map of CCA (Supplementary Tables S6 and S7 for raw data). One significant canonical variable was extracted in Maremmana breed, with a canonical correlation coefficient of 0.99, whereas two canonical variables were extracted in Aubrac breed, with canonical correlation coefficients of 0.98 and 0.94 for the first and the second canonical variables, respectively. The relationships between the OCFAs and BCFAs and bacterial genera are mapped in Fig. 3A and B for Maremmana and Aubrac, respectively. In Maremmana breed, Prevotellaceae_UCG-003, Ruminococcaceae_UCG-010, and *Oribacterium* showed a positive correlation with C13:0iso and C17:0. Conversely, in Aubrac, Prevotellaceae_UCG-001 and Ruminococcus_1 correlate positively with C14:0iso, C15:0, C15:0iso, C17:0, and negatively with C13:0.

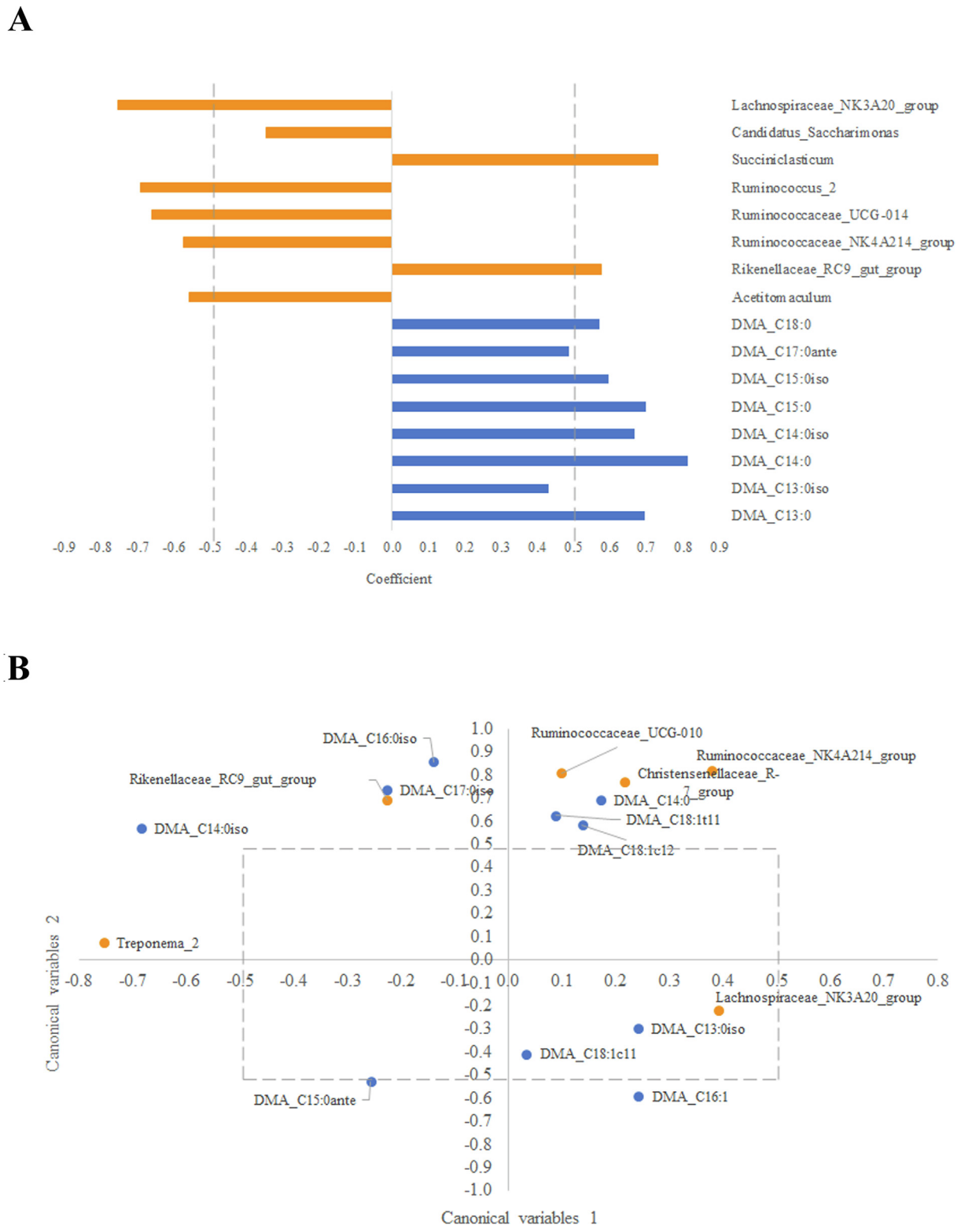

Fig. 1. Canonical correlation analysis (CCA) similarity maps for the bacterial genera and dimethylacetals (DMAs) in Maremmana (A) and Aubrac (B).

Saccharofermentans, Rikenellaceae_RC9_gut_group, Ruminococcaceae_NK4A214_group, and Ruminococcaceae_UCG-010 correlate positively with C13:1c12 and C16:0iso. For Maremmana, the raw variance of the OCFAs and BCFAs variables explained by the genera variables (redundancy analysis) was 26%, whereas for Aubrac, it was 68%.

Discussion

Discriminant analysis

Several studies revealed a high inter-animal variation and a low intra-animal variation among rumen bacterial communities

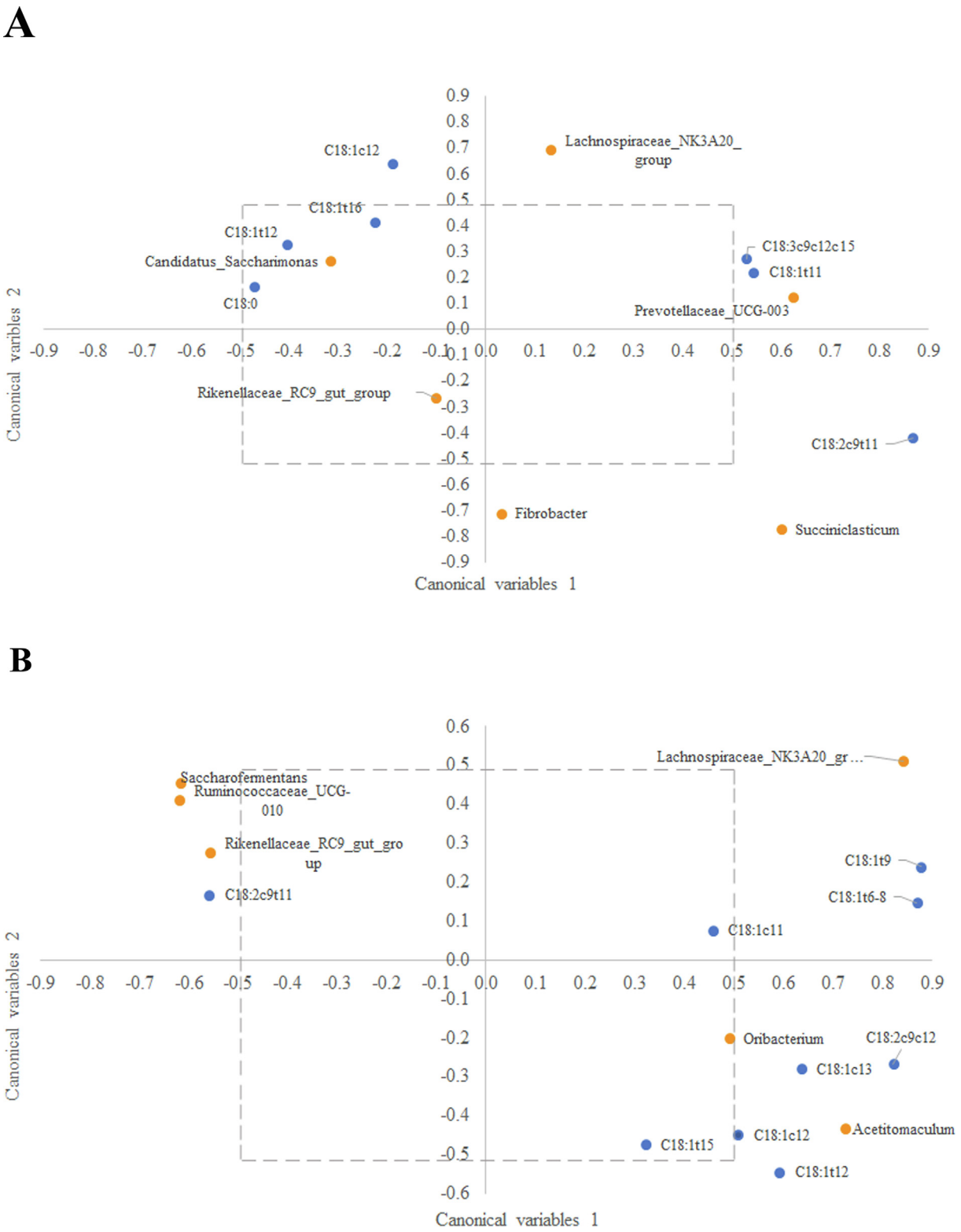

Fig. 2. Canonical correlation analysis (CCA) similarity maps for the bacterial genera and fatty acids involved in biohydrogenation (BHFAs) in Maremmana (A) and Aubrac (B).

(Mullins et al., 2013). It has been suggested that differences in the bacterial communities among different animals may be related to feed selection/sorting, feeding and drinking behaviour, rumination time and feed passage rate in the rumen (McCann et al., 2014). Moreover, it was discovered that there are differences in the rumen bacterial populations between different breeds of beef (Hernandez-Sanabria et al., 2013; Daghio et al., 2021) and dairy cattle (Bainbridge et al., 2016), suggesting that host genetics may impact the community structure and rumen bacterial diversity. For this reason, our investigation started with a CDA, to verify if the bacterial population and the rumen lipid metabolism interact differently in the two breeds studied. The results of CDA encourage

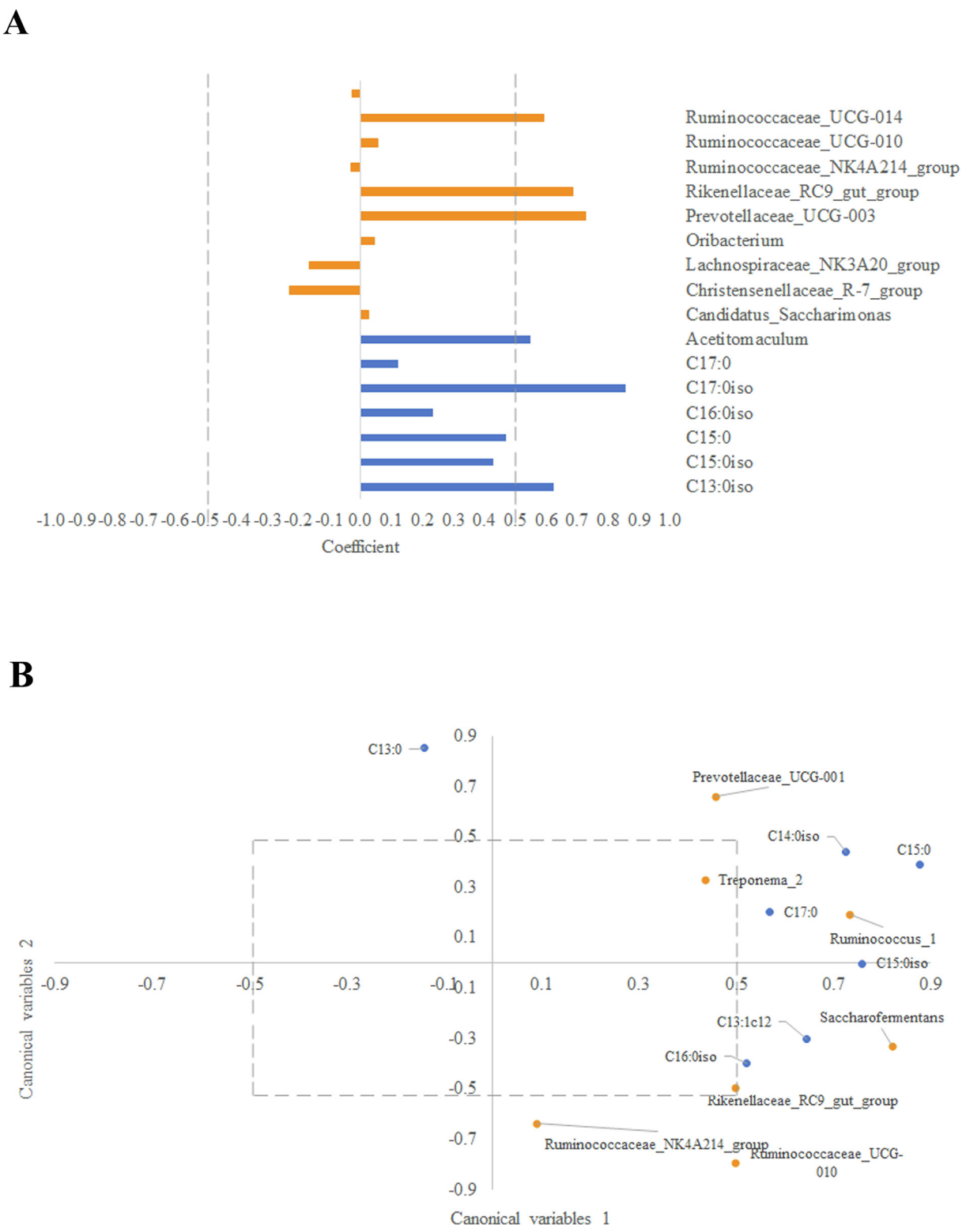

Fig. 3. Canonical correlation analysis (CCA) similarity maps for the bacterial genera and odd and branched fatty acids (OCFAs and BCFAs) in Maremmana (A) and Aubrac (B).

to hypothesise that Maremmana and Aubrac may be discriminated both from the point of view of the bacterial population composition and of lipid metabolism products. On the contrary, there was no clear discrimination between the two farming systems (feedlot and grazing). This result is in agreement with what was observed in a previous work, where the effect of the farming system on the profile of fatty acids and on the composition of rumen bacterial population was not detected (Daghio et al., 2021). Regarding the fatty acid profile, the two breeds can be discriminated by considering the concentration of DMAs, OCFAs, BCFAs and the biohydrogenation process products. The perfect discrimination between Maremmana and Aubrac reared in same farming system leads to think that the two breeds showed a different relationship with the rumen bacterial population, which also affects

the rumen lipid metabolism. This aspect would justify the preponderant role of ruminal symbiosis which does not lead to a complete discrimination of the effect of the breeding system. These findings are in accordance with previous studies which demonstrated that fatty acids may be important markers of rumen microbial metabolism characterising the host genetic effect (Mosley et al., 2002; Bainbridge et al., 2016). The results of the CDA show that in this study, a different number of variables were necessary to discriminate the two breeds according to the dataset used: 6, 5, 12 and 11 for DMAs, OBCFAs, BHFAs and bacterial genera, respectively. Generally, the lower the number of variables contributing to the discrimination, the sharper the difference among groups (De Maesschalck et al., 2000). In this study, animals of the two breeds were fed the same diet which did not explain the differences observed. In CDA fewer DMAs, OCFAs and BCFAs were enough to discriminate between the two breeds, while a higher number of BHFAs was needed. The profile of these fatty acids depends on the quality of animal diet which affects the profile of microbial community (Buccioni et al., 2012). Hence, DMAs, OCFAs, BCFAs and BHFAs together with the abundance of 11 bacterial groups were needed to highlight the possible discriminating effect.

Canonical correlation analysis

Data were further analysed using CCA to study the potential relationship among bacterial genera, DMAs derived from plasmalogenic acids, OCFAs, BCFAs, BHFA products and to assign a more specific role to the bacterial population in rumen lipid metabolism. As a consequence of the high number of identified bacteria and of markers considered, it was preferable to use a multivariate approach to simplify the study phenomenon interpretation. Therefore, a CCA was used for each pathway studied, relating the level of rumen bacteria with final and/or intermediate products of biohydrogenation, OCFAs and BCFAs synthesis and DMAs. The CCA is a common characteristic extraction method in multivariate statistical analysis, which provides an efficient way of measuring the linear relationship between two multidimensional variables (Dai et al., 2015; Fan et al., 2018). However, the number of variables is too large for the application of the CCA, due to the introduction of useless information that can increase the risk of overfitting (Dai et al., 2015; Fan et al., 2018). Therefore, CCA was carried out on the dataset of the most influential variables established on the basis of those that showed high and significant correlations (Pearson correlation greater than 0.6). As demonstrated by redundancy indices, bacterial community profile affected DMA, BHFA and OCFA and BCFA profiles. Redundancy indices represent the level of variance shared by a group of variables (in our work DMAs, BHFAs, OCFAs and BCFAs) as explained by the canonical variable of the other group of variables (in our study, bacterial genera). A high level of redundancy indices ranging from 24% to 68% of variance was found in the dependent variables (DMAs, OCFAs and BCFAs and BHFAs). This result was explained by the independent canonical variable (bacterial genera). In addition, the averages of the shared variances explained by the dependent and independent canonical variables were 10.5% and 18.7%, respectively. Redundancy indices higher than 20% are considered a good level to highlight the strict relationship between two sets of variables (Stürmer et al., 2018).

Canonical correlation analysis between bacteria taxa and dimethylacetals

The DMAs are derivatives of plasmalogen lipids, which are found in many strictly anaerobic bacteria, but are not present in aerobic or facultative anaerobic bacteria (Goldfine, 2010). They affect the membrane fluidity of gram-negative bacteria. In particular, DMA profile has been proposed as potential marker of the response of rumen bacteria to the environmental conditions (Katz and Keeney, 1964; Minato et al., 1988; Goldfine, 2010). Literature shows that DMA profile may be interpreted as the expression of bacterial resilience to changes occurred in the environment in which they live. Hence, it is related either to the feeding strategy (feed ingredients, feed particle dimensions that influence the rate of feed passage in the rumen, ingestion level that affects the rumen microbial community) (Cappucci et al., 2018; Cappucci et al., 2021) or to animal species. In Maremmana, the result of CCA revealed a positive relation of *Succiniclasticum* and of a member of the family *Rikenellaceae* (i.e. Rikenellaceae_RC9_gut_group) with the level of $DMA_{C18:0}$, $DMA_{C15:0iso}$, $DMA_{C15:0}$, $DMA_{C14:0iso}$, $DMA_{C14:0}$ and $DMA_{C13:0}$. Rikenellaceae_RC9_gut_group has shown a close correlation with several DMAs ($DMA_{C14:0iso}$, $DMA_{C17:0iso}$ and $DMA_{C16:0iso}$) in Aubrac as well. Notably, $DMA_{C14:0iso}$ showed a close correlation with Rikenellaceae_RC9_gut_group in both breeds. This taxon was positively correlated to the growth performances of host animal and is involved in the production of short-chain fatty acids, especially of propionate. The synthesis of propionate from pyruvate leads to more efficient energy recovery and low methane emission due to a lack of H_2 production in this metabolic pathway and to the sink of H during the oxidation of NADH and $FADH_2$ (Holman and Gzyl, 2019; Tong et al., 2018). In our findings, the presence of high levels of iso-branched DMAs, in particular $DMA_{C14:0iso}$, was associated with the increased presence of Rikenellaceae_RC9_gut_group whose abundance was higher in animals characterised by the best growth performances (Daghio et al., 2021). This association is very interesting and further investigations are needed to verify if Rikenellaceae_RC9_gut_group may play a role in affecting the productivity of ruminants. Nowadays, no information is available in literature on this association.

In Aubrac, a close association of Treponema_2 with Rikenellaceae_RC9_gut_group was found. Considering that these two genera were also associated to the synthesis of $DMA_{C14:0iso}$, $DMA_{C17:0iso}$ and $DMA_{C16:0iso}$, a symbiotic relationship between these two genera could be hypothesised. Indeed, literature reported that Treponema_2 is a common rumen bacterial group ascribed in the fibre degradation metabolism (Shinkai et al., 2010; Bekele et al., 2010) and it is also reported that the synthesis of iso fatty acids is related to the cellulosolytic bacteria activities (Vlaeminck et al., 2006).

Lachnopiraceae_NK3A20_group, Ruminococcus_2 5 Ruminococcaceae_UCG-014, and Ruminococcaceae_NK4A214_-group showed an opposite effect in Maremmana than Aubrac, as reported by Daghio et al. (2021). These bacterial taxa are members of the *Lachnospiraceae* and *Ruminococcaceae* families that are extensively recognised as members of the core rumen microbiota (≥90% of samples) (Henderson et al., 2015; Mannelli et al., 2019). All these groups showed a higher abundance in high production cows, so high level of $DMA_{C18:0}$, $DMA_{C15:0iso}$, $DMA_{C15:0}$, $DMA_{C14:0iso}$, $DMA_{C14:0}$ and $DMA_{C13:0}$ may be related to a lower level of these taxa and to lower animal performance (Tong et al., 2018). In Aubrac, other bacteria, belonging to *Firmicutes* (Ruminococcaceae_UCG-010, Ruminococcaceae_NK4A214_group and Christensenellaceae_R-7_group), are correlated with $DMA_{C18:1t11}$, $DMA_{C14:0}$ and $DMA_{C18:1c12}$. In the gastrointestinal environment, members of the *Lachnospiraceae*, *Ruminococcaceae* and *Christensenellaceae* were able to degrade plant cellulose and hemicellulose, and convert them into short-chain fatty acids that can be absorbed and used for energy by host (Wang et al., 2019). Ruminococcus_2 and Ruminococcaceae_NK4A214_group were present in higher relative abundances in rumen liquor from animal fed forage compared to those in rumen liquor from animal fed concentrate (Ferrario et al., 2017). Interestingly, the CCA revealed that amylolytic bacteria (e.g. Rikenellaceae_RC9_gut_group) are characterised by a higher level of DMAs with iso-branched-chain fatty acids, while cellulolytic bacteria are related to DMAs with fatty

acids deriving from biohydrogenation (C18:1t11 and C18:1c12). Nowadays, literature gives many evidences which confirm the involvement of several cellulolytic bacteria strains in biohydrogenation processes (Dehority, 2003; Jenkins et al., 2008) while amylolytic bacteria are usually more frequently associated to anteiso fatty acids than iso fatty acids (Vlaeminck et al., 2006). Hence, further investigations are needed to better explain the relationship between amylolytic bacteria and DMAs derived from isobranched-chain fatty acids and to deepen the knowledge on the role of cellulolytic bacteria in biohydrogenation pathways.

Canonical correlation analysis between bacteria taxa and biohydrogenation fatty acids

Rumen microorganisms may use biohydrogenation as protection against unsaturated fatty acid toxicity (Buccioni et al., 2012). Even between bacteria and BHFAs, the CCA found a close correlation that permitted to make inference to biohydrogenation process. Interestingly, Lachnospiraceae_NK3A20_group showed a close correlation with BHFAs in both breeds, although with different fatty acids. In Maremmana, Lachnospiraceae_NK3A20_group and Prevotellaceae_UCG-003 are related to C18:3c9c12c15 (α-linolenic acid) and C18:1t11. In contrast, Lachnospiraceae_NK3A20_group is associated with C18:1t9 and C18:1t6-8 in Aubrac. These two fatty acids can be produced by rumen biohydrogenation of oleic acid present in animal feed (Mosley et al., 2002, 2006; Toral et al., 2018). The family*Lachnospiracea* is one of the main component of the gastrointestinal microbiota of ruminants. They play an important role in rumen development being butyric acid producers and promoting the increase of rumen epithelial papilla length, width, and surface. The optimal development of rumen improves the absorption and digestion of nutrients in host animal that get more energy and accelerate the growth. The different behaviours of this member of the family *Lachnospiraceae* in the two breeds may be due to a perturbation linked to the composition of the diet (differences due to behaviour in the intake of feeds, to ingestion capacity, to voluntary consumption or to rate of rumen flow) and how this is fermented at the rumen level (Pallara et al., 2014).

The data-related associations found in this study seem to confirm the involvement of Lachnospiraceae_NK3A20_group in the biohydrogenation of C18:2c9c12 and α-linolenic acid in both breeds. This is plausible because the family of *Lachnospiraceae* harbours many bacterial species involved in different rumen metabolic pathways included the biohydrogenation of PUFAs (Pallara et al., 2014). Considering the biohydrogenation pathway, it could be hypothesised that biohydrogenation of α-linolenic acid and linoleic acid in Maremmana follows the way of C18:1t11 that represents the principal biohydrogenation intermediate product (Buccioni et al., 2012), while the biohydrogenation of C18:2c9c12 in Aubrac follows the alternative pathway through C18:1t9 and C18:1t6-8 (Shingfield et al., 2010; Huws et al., 2011; Shingfield and Wallace, 2014). However, more investigation is needed because data reported in this study are not enough to confirm this speculation. In Maremmana, breed Prevotellaceae_UCG-003 showed greater activity in the presence of forage (Van Gylswyk, 1995) and could be related to forage α-linolenic acid biohydrogenation. However, this correlation was not observed in the Aubrac breed, despite fed the same diet. These differences could be due to breed characteristics as ingestion capacity, rumen flow and feeding rhythm. The Maremmana is an ancient local breed whose feeding behaviour is characterised by high consumption of roughage (more than Aubrac). This feature probably has contributed to develop the ability in fermenting fibrous diet more efficiently (Conte et al., 2019), favouring a higher activity of cellulosolytic bacteria. A close relationship of *Fibrobacter* and *Succiniclasticum* with C18:2c9t11 (rumenic acid) was found (Daghio et al., 2021). The presence of genus *Succiniclasticum* is widely described in rumen communities (Holman and Gzyl, 2019; Auffret et al., 2017). This genus is involved in the production of propionate, starting from succinate (van Gylswyk, 1995). Furthermore, the level of its activity is positively correlated to animal feed efficiency (Auffret et al., 2017; Clemmons et al., 2020), since propionate is the main precursor of gluconeogenesis (Shabat et al., 2016). In contrast, the genus *Fibrobacter* is involved in the conversion of cellulose to succinate that can be used as a substrate for the production of propionate by the genus *Succiniclasticum* (Holman and Gzyl, 2019). The close correlation of these two bacterial genera, observed in the CCA, suggests that a possible metabolic interaction exists within the metabolism of succinate. As emerges from the CCA, the joint activity of *Fibrobacter* and *Succiniclasticum* is strongly associated with the production of rumenic acid during biohydrogenation and an interesting association was revealed between *Acetitomaculum* and LA, C18:1t12, C18:1c12 and C18:1c13. This genus is characterised by an acetogenic activity (Angelidaki et al., 2003), which is a pathway associated to a consumption of H_2 and to a lowering of methanogenesis (Gagen et al., 2015). The growth of *Acetitomaculum* is strongly enhanced by high-forage diets. This condition is also largely advantageous for the growth of cellulolytic bacteria, most of them engaged in biohydrogenation of PUFAs. Hence, the association between *Acetitomaculum* and the biohydrogenation intermediates found in this study could be explained by the favourable growth conditions at the same time for this microorganism and for cellulolytic bacteria or by a possible direct role of *Acetitomaculum* in biohydrogenation process. However, our data did not allow to discern which of the two hypotheses can be supported.

Canonical correlation analysis between bacteria taxa and odd and branched fatty acids

Generally, OCFAs and BCFAs are considered as microbial markers in the rumen ecosystem (Kaneda, 1991, Vlaeminck et al., 2006; Bessa et al., 2009) because they are synthesised by rumen bacteria, which used them to modulate the fluidity of their membranes, according to a lower melting point than their straight-chain counterparts (Suutari and Laakso 1992; Poger et al., 2014). Thus, rumen OCFA and BCFA profile may reflect the relative abundance of bacterial populations as reported by Vlaeminck et al. (2006). In this trial, the most abundant OCFAs and BCFAs are C15:0, C15:0iso and C15:0ante. The first two fatty acids showed a high correlation with Ruminococcus_1 and Prevotellaceae_UCG-001 in Aubrac. Previous *in vitro* studies are consistent with our findings because demonstrated that the relative abundance of *Prevotellaceae* tend to increase with C15:0iso as a consequence of their cellulolytic activities (Logar et al., 2001; Vlaeminck et al., 2006). Another cellulolytic bacterium associated with C15:0 and C15:0iso was the genus Ruminococcus_1 that showed a strict relationship with Prevotellaceae_UCG-001. A mutual relationship between these genera could be hypothesised. A very interesting correlation was that between C13:1c12 and the genus *Saccharofermentans*. This fatty acid needs further investigations because its presence in the rumen fluid is not recognised by most of the works and therefore its role is rather controversial. Two trials (Tahoun et al., 1986; Mishina et al., 1977) demonstrated that some fungi synthesise C13:1c12 when they are in the presence of sugars, in particular galactose, fructose and sucrose. These works hypothesise that fungi can also synthesise this fatty acid to regulate membrane fluidity. The correlation of C13:1c12 with *Saccharofermentas* could suggest a linkage between C13:1c12 and the metabolism of carbohydrates such as sucrose. However, additional investigations are necessary to demonstrate this hypothesis.

Conclusions

This study showed that Maremmana and Aubrac rumen microbial community differentiate regardless of dietary regimen suggesting that the effect of host genetic in the regulation of rumen metabolism is not negligible. However, further investigation are needed to verify if the assumptions made on the base of the findings in these trials may be confirmed.

The combined CDA and CCA multivariate statistical analysis approach may represent an efficient method to investigate the association among a particular bacterial genus and specific products of lipid metabolism. Thus, these relationships could be an interesting tool in the interpretation of rumen lipid metabolism, and in the definition of microbial community profile changes.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100520.

Ethics approval

All experiments in this study were performed in accordance with the approved guidelines from the European directive 2010/63/UE and DL 4/03/2014 no. 26.

Data and model availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, BioProject number PRJNA682716, BioSample accession numbers SAMN17005974-SAMN17006013.

Data not available online are available from authors upon request.

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Author contributions

GC, AS, AB, CV and MM conceived and designed the research. GC and CD wrote the manuscript. MD performed the bioinformatic analysis. GC and AS performed the field sampling. GC and CD performed the statistical analysis. GC, FM, AM and AB performed the chemical analyses. BM and JV performed the amplicon sequencing. AB, CV, JV and MM reviewed the manuscript. All authors read and approved the submitted version.

Declaration of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgements

The authors sincerely thank the companies "Tenuta di Paganico Soc. Agr. Spa", "Podere dei Fiori Soc. Agr." and "Pietratonda soc. Agr.", who followed the animals throughout the test.

Financial support statement

This work was performed in the framework of the PROSMART-BEEF project, Rural Development Programme, Tuscany region, 2014–2020, 16.2 measure.

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