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Effect of stoned olive pomace on rumen microbial communities and polyunsaturated fatty acids biohydrogenation: an *in vitro* study

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Abstract

Background

The stoned olive pomace (SOP), which represents approximately 50% of the conversion process of olive in oil, is largely not-utilized and creates costs for its disposal and problems related to environmental impact. *In-vitro* trial experiments were employed to study the effect of feeds integrated with this bio-waste rich in polyphenols on rumen biohydrogenation, using sheep rumen liquor as inoculum.

Results

Fatty acid (FA) analysis and polymerase chain reaction denaturing gradient gel electrophoresis (DGGE) approach aimed to microbial community characterization showed that including SOP in feeds at the level of 50 g/kg and 90 g/kg induced changes in FA profile and in microbial populations. The contemporary decrease of *Butyrivibrio proteoclasticus* and the accumulation of vaccenic acid was observed. A depression of *Neisseria weaveri*, *Ruminobacter amylophilus* and other unclassified bacteria related to members of the *Lachnospiraceae* and *Pasteurellaceae* families was detected, suggesting that these microbial groups may be involved in rumen biohydrogenation.

Conclusions

Supplementation of feeds with SOP changes rumen bacterial community, including bacteria responsible for vaccenic acid hydrogenation to stearic acid, thus modifying FA profile of rumen liquor. Hence, an use of SOP aimed to produce meat or dairy products enriched in functional lipids could be hypothesized.

Keywords

Stoned olive oil pomace, Sheep rumen microbiota, Fatty acid biohydrogenation, PCR-DGGE

Background

The main factor affecting ruminal biohydrogenation (BH) is animal diet, whose quality influences the content of healthful fatty acids (FA) in milk and meat. The inclusion of polyphenols in ruminant feeds has an inhibitory effect on BH of dietary PUFA, as consequence of their influence on microbial activity and diversity [1]. This has been shown to increase duodenal flow of bioactive FA, as vaccenic (*trans*-11 C18:1, VA) and, as consequence, to improve the nutritional value of milk fat from large and small dairy

ruminants, since this FA can be Δ^9-desaturated to rumenic acid (*cis*-9,*cis*-12 C18:2, RA) in the mammary gland and in other tissues. However, from literature it is well known that the availability of VA in ruminant products is limited by its hydrogenation to stearic acid (C18:0, SA) or isomerization to other C18:1 isomers by microbial activity taking place in the rumen [2].

Rumen microbial community comprises an enormous number of microbial species belonging to Bacteria, Archaea and Eucarya domains. However, only a limited number of them has been isolated and characterized physiologically until now [3]. Among the different microbial species already identified, those ones belonging to the *Butyrivibrio* group appear of particular interest, because they are known to be directly involved in BH [4]. Recent studies carried out on different species of ruminants have reported that diets enriched with polyphenols caused a decrease in SA and a contemporary decline of key species of *Butyrivibrio* [1,5]. In last few years there has been also an arising interest on the impact of polyphenols on the methanogenic community, which largely contributes to the emissions of greenhouse gas to the atmosphere [6].

In Mediterranean area, during the period of olive oil production, there is an high production of moist virgin olive pomace that represents a problem for its disposal. The most recent stoning virgin olive pomace techniques permit to obtain a residual product (stoned olive pomace, SOP) characterized by a good level of polyphenols and low lignin content, which is considered the main factor reducing digestibility of olive pomace or olive cake when these by-products are utilized as animal feed [7-9]. These chemical characteristics make SOP potentially able to interfere with rumen fermentation [7,10]. However, little information is available in literature on the effect of SOP inclusion in ruminant diets on microbial strains involved in BH processes and methanogenesis. The aim of the present study was to verify whether SOP supplementation in sheep diet is able to affect both the overall rumen microbial profile and specific rumen microbial groups with particular regard to the *Butyrivibrio* group and methanogenic archeal populations, thus influencing the PUFA profile of rumen liquor (RL).

Results

Rumen liquor fatty acid composition

During the fermentation of the three feeds, the concentration of acetic acid (C2:0) did not show significant differences with the exception of 12 h, when the percentage of this volatile fatty acid was higher in RL fermenting S5 and S9 (Table 1). Moreover, the presence of SOP in feeds significantly increased the concentration of propionic (C3:0), butyric (C4:0) and iso-valeric (*iso* C5:0) acids compared to the content of these FA in RL with C. As consequence, the ratio C2:0/C3:0 in S5 and S9 was significantly lower than that in RL fermented with C at 12 and 24 h (Table 1).

Table 1 Effect of stoned olive pomace (SOP) concentration (mM) on volatile fatty acid (VFA) production in ruminal fluid at 6, 12 and 24 h of incubation[1]

VFA	Feed	Time (h)			SEM	P
		6	12	24		
C2:0	C	5.690 $^{a\alpha}$	7.360 $^{b\alpha}$	9.370 ^{c}	0.240	0.021
	S5	6.700 $^{a\beta}$	8.020 $^{b\beta}$	9.190 ^{c}		
	S9	3.690 $^{a\gamma}$	8.530 $^{b\beta}$	9.200 ^{c}		
C3:0	C	2.450 $^{\alpha}$	2.670 $^{\alpha}$	3.010 $^{\alpha}$	0.340	0.047
	S5	3.090 $^{\alpha\beta}$	3.250 $^{\alpha\beta}$	3.790 $^{\alpha\beta}$		
	S9	3.150 $^{\beta}$	3.990 $^{\beta}$	4.230 $^{\beta}$		
C4:0	C	3.050 $^{a\alpha}$	3.490 $^{b\alpha}$	3.840 $^{c\alpha}$	0.060	0.042
	S5	3.450 $^{a\beta}$	3.750 $^{b\beta}$	4.020 $^{c\beta}$		
	S9	3.670 $^{a\gamma}$	3.980 $^{b\gamma}$	4.150 $^{b\beta}$		
iso C4:0	C	0.156	0.189	0.193	0.075	0.083
	S5	0.135	0.147	0.153		
	S9	0.114	0.113	0.112		
C5:0	C	0.165	0.196	0.264	0.081	0.079
	S5	0.194	0.217	0.210		
	S9	0.105	0.233	0.235		
iso C5:0	C	0.350 $^{a\alpha}$	0.360 $^{a\alpha}$	0.500 $^{b\alpha}$	0.030	0.037
	S5	0.460 $^{a\beta}$	0.600 $^{b\beta}$	0.680 $^{c\beta}$		
	S9	0.490 $^{a\beta}$	0.800 $^{b\gamma}$	0.820 $^{b\gamma}$		
C2/C3	C	2.322 $^{a\alpha}$	2.756 $^{\alpha ab}$	3.113 $^{b\alpha}$	0.292	0.048
	S5	2.168 $^{\alpha}$	2.68$^{\alpha\beta}$	2.424 $^{\alpha}$		
	S9	1.171 $^{a\beta}$	2.138$^{\beta b}$	2.175 $^{b\beta}$		

α,β,γ Within a column, means with different Greek superscripts are significantly different ($P < 0.05$); a,b,c within a row, means with different Latin superscripts are significantly different ($P < 0.05$). C = control feed; S5 = treatment with 50 g/kg of SOP; S9 = treatment with 90 g/kg of SOP.

[1]Mean values with their standard errors (SEM); number of samples for each treatment at any time =3.

C14:0 and C16:0 increased in rumen fluid incubated with S5 while with C and S9 the concentration of these FA significantly decreased (Table 2). S5 and S9 significantly increased the C13:0 concentration within 12 h but at 24 h the percentage of this FA was significantly lower than that found in fermenters containing C (Table 2). Moreover at 24 h C15:0 percentage was the highest in S5 and C fermenters. C17:0 production was significantly depressed by S9 but not by S5, that resulted similar to C (Table 2).

Table 2 Effect of stoned olive pomace (SOP) concentration (g/100g total fatty acids, FA) on medium chain fatty acid production in ruminal fluid at 6, 12 and 24 h of incubation[1]

FA	Feed	Time (h)			SEM	P		FA	Feed	Time (h)			SEM	P	
		6	12	24		F	FxT			6	12	24		F	FxT
C12:0	C	0.507 $^{a\alpha}$	0.447 ^{ab}	0.357 $^{b\alpha}$	0.048	0.069	0.027	*anteiso* C15	C	0.510 $^{\alpha}$	0.570 $^{\alpha}$	0.523 $^{\alpha}$	0.020	0.996	0.049
	S5	0.403 $^{ab\beta}$	0.363 ^{b}	0.443 $^{a\alpha\beta}$					S5	0.477 $^{a\alpha}$	0.490 $^{a\beta}$	0.626 $^{b\beta}$			
	S9	0.553 $^{a\alpha\beta}$	0.403 ^{b}	0.320 $^{c\beta}$					S9	0.700 $^{a\beta}$	0.463 $^{b\gamma}$	0.443 $^{b\gamma}$			
C13:0	C	0.973 $^{a\alpha}$	1.133 $^{a\alpha}$	1.676 $^{b\alpha}$	0.041	0.791	0.034	*iso* C16	C	0.143 $^{\alpha}$	0.147	0.123 $^{\alpha}$	0.020	0.199	0.027
	S5	1.117 $^{a\alpha}$	1.360 $^{b\beta}$	1.340 $^{b\beta}$					S5	0.106 $^{a\beta}$	0.147 ^{b}	0.173 $^{c\beta}$			
	S9	0.640 $^{a\beta}$	1.526 $^{b\gamma}$	1.463 $^{b\gamma}$					S9	0.146 $^{a\alpha}$	0.169 ^{b}	0.186 $^{c\beta}$			
C14:0	C	0.847 $^{a\alpha}$	0.823 $^{a\alpha}$	0.753 $^{b\alpha}$	0.030	0.874	0.012	*iso* C17	C	0.110	0.136 $^{\alpha}$	0.133 $^{\alpha}$	0.030	0.002	0.044
	S5	0.663 $^{a\beta}$	0.730 $^{a\beta}$	0.913 $^{b\beta}$					S5	0.123 ^{a}	0.183 $^{b\beta}$	0.176 $^{b\alpha\beta}$			
	S9	0.930 $^{a\gamma}$	0.796 $^{b\beta}$	0.593 $^{c\gamma}$					S9	0.107 ^{a}	0.183 $^{b\beta}$	0.193 $^{b\beta}$			
C16:0	C	5.440 $^{a\alpha}$	5.063 $^{b\alpha}$	4.957 $^{b\alpha}$	0.058	0.059	0.039	*anteiso* C17	C	0.143 $^{\alpha\beta}$	0.173	0.174 $^{\alpha}$	0.007	0.041	0.042
	S5	4.570 $^{a\beta}$	4.740 $^{b\beta}$	5.537 $^{c\beta}$					S5	0.116 $^{a\alpha}$	0.133 ^{a}	0.201 $^{b\alpha}$			
	S9	5.780 $^{a\alpha}$	4.327 $^{b\gamma}$	3.860 $^{c\gamma}$					S9	0.177 $^{a\beta}$	0.133 ^{a}	0.101 $^{b\beta}$			
C17:0	C	0.080 $^{a\alpha}$	0.093 ^{a}	0.127 $^{b\alpha}$	0.020	0.943	0.048	C12:1	C	0.040 $^{a\alpha}$	0.047 $^{a\alpha}$	0.013 $^{b\alpha}$	0.030	0.061	0.002
	S5	0.073 $^{a\alpha}$	0.103 ^{b}	0.120 $^{b\alpha}$					S5	0.017 $^{a\beta}$	0.036 $^{b\alpha}$	0.050 $^{c\beta}$			
	S9	0.113 $^{a\beta}$	0.089 ^{b}	0.088 $^{b\beta}$					S9	0.037 $^{a\alpha}$	0.020 $^{b\beta}$	0.030 $^{ab\gamma}$			
iso C13	C	0.081	0.111 $^{\alpha}$	0.103 $^{\alpha}$	0.020	0.610	0.048	C14:1	C	0.277 $^{a\alpha}$	0.353 $^{b\alpha}$	0.287 $^{a\alpha\beta}$	0.016	0.077	0.047
	S5	0.086	0.081 $^{\alpha}$	0.087 $^{\alpha}$					S5	0.233 $^{a\alpha\beta}$	0.260 $^{a\beta}$	0.363 $^{b\beta}$			
	S9	0.091^{a}	0.173 $^{b\beta}$	0.167 $^{b\beta}$					S9	0.333 $^{a\beta}$	0.273 $^{ab\beta}$	0.250 $^{b\alpha}$			
iso C14	C	0.087 $^{\alpha}$	0.087	0.097 $^{\alpha}$	0.010	0.497	0.015	C15:1	C	0.077 $^{\alpha}$	0.076	0.053 $^{\alpha}$	0.030	0.046	0.046
	S5	0.077 $^{a\alpha\beta}$	0.091 ^{a}	0.130 $^{b\beta}$					S5	0.053 $^{a\beta}$	0.056 ^{a}	0.093 $^{b\beta}$			
	S9	0.110 $^{a\alpha}$	0.081 ^{b}	0.081 $^{b\alpha}$					S9	0.100 $^{a\alpha}$	0.057 ^{b}	0.093 $^{a\beta}$			
iso C15	C	0.076 $^{a\alpha}$	0.073 $^{a\alpha\beta}$	0.037 $^{b\alpha}$	0.010	0.275	0.047	C16:1	C	0.087	0.116	0.103	0.016	0.051	0.049
	S5	0.053 $^{a\beta}$	0.080 $^{b\beta}$	0.076 $^{b\beta}$					S5	0.167	0.149	0.133			
	S9	0.073 $^{\alpha}$	0.057 $^{\alpha}$	0.060 $^{\beta}$					S9	0.123	0.100	0.093			

α,β,γ Within a column, means with different Greek superscripts are significantly different (P<0.05); a,b,c within a row, means with different Latin superscripts are significantly different (P<0.05). C = control feed; S5 = treatment with 50 g/kg of SOP; S9 = treatment with 90 g/kg of SOP; F, FxT: probability of significant effect due to experimental factors: feeds (F) and the interaction feed x time (FxT).

[1]Mean values with their standard errors (SEM); number of samples for each treatment at any time = 3.

At the last point of sampling the concentration of *iso* C15, *iso* C16 and *iso* C17 was significantly higher in S5 and S9 fermenters than in C (Table 2). Respect to C, *anteiso* C15 content was depressed during the fermentation of S9 and enhanced when S5 was fermented (Table 2). Moreover, the content of C17 *ante* increased during the fermentation of S5, whereas S9 showed an opposite trend (Table 2). The concentration of C12:1, C14:1 and C15:1 was characterized by an increasing trend in fermenters containing S5 (Table 2). The content of C18:0 increased during the whole time of fermentation regardless the treatment but with a lower extent for fermenters containing SOP in a dose dependent manner (Table 3). When RL was incubated with S5, *cis*-11 C18:1 and *cis*-13 C18:1 increased significantly after 12 h compared to the fermenters containing C and S9 (Table 3). Moreover, S5 decreased significantly the BH rate of *cis*-9 C18:1, that at 24 h showed the highest concentration (Table 3). VA was progressively accumulated during the whole period of fermentation when SOP is added to feeds regardless the percentage of inclusion, as consequence of a decrease of BH extent (Table 3). No significant differences among feeds were found for the other *trans* monoenes (Table 3). RA was accumulated at 12 h in any case but, when S5 and S9 were fermented, the its percentage in RL was the highest according to a decrease of BH rate (Table 3). In contrast, *trans*-10,*cis*-12 C18:2 was detected only at 12 h in S5 fermenters (Table 3). The BH rate of linoleic (*cis*-9,*cis*-12 C18:2, LA) and α-LNA (*cis*-9,*cis*-12,*cis*-15 C18:3) acids was similar in C and S9 (Table 3). In S5 it was simply lowered leading to a higher accumulation of LA and α-LNA at 24 h. Conjugated linolenic acid (*cis*-9,*trans*-11,*cis*-15 C18:3) and vaccelenic acid (*trans*-11,*cis*-15 C18:2) were detected at 24 h only in S9 fermenters (Table 3).

Table 3 Effect of stoned olive pomace (SOP) concentration on C18 fatty acids (g/100 g FA) production in ruminal fluid at 6, 12 and 24 h of incubation[1]

FA	Feed	Time (h)			SEM	P	FA	Feed	Time (h)			SEM	P
		6	12	24					6	12	24		
C18:0	C	2.377 $^{a\alpha}$	2.590 $^{b\alpha}$	4.167 $^{c\alpha}$	0.047	0.006	*trans*-11 C18:1	C	0.580 ^{a}	0.350 $^{b\alpha}$	0.120 $^{c\alpha}$	0.021	0.005
	S5	2.203 $^{a\beta}$	3.080 $^{b\beta}$	3.446 $^{c\beta}$				S5	0.483 ^{a}	0.677 $^{b\beta}$	0.850 $^{c\beta}$		
	S9	1.760 $^{a\gamma}$	2.806 $^{b\gamma}$	2.999 $^{c\gamma}$				S9	0.473 ^{a}	0.653 $^{b\beta}$	0.830 $^{c\beta}$		
cis-9 C18:1	C	2.063 $^{a\alpha}$	1.610 $^{b\alpha}$	1.290 $^{c\alpha}$	0.023	0.032	*trans*-12 C18:1	C	0.047	0.047	0.053	0.024	0.485
	S5	1.950 $^{a\beta}$	1.526 $^{b\beta}$	1.567 $^{c\beta}$				S5	0.056	0.050	0.043		
	S9	2.563 $^{a\gamma}$	1.663 $^{b\gamma}$	1.163 $^{c\gamma}$				S9	0.053	0.036	0.029		
cis-11 C18:1	C	0.437	0.420	0.473	0.068	0.046	*cis*-9,*cis*-12 C18:2	C	4.527 $^{a\alpha}$	3.180 $^{b\alpha}$	1.733 $^{c\alpha}$	0.043	0.031
	S5	0.340 ^{a}	0.360 ^{a}	0.527 ^{b}				S5	3.750 $^{a\beta}$	2.653 $^{b\beta}$	2.060 $^{c\beta}$		
	S9	0.487	0.350	0.363				S9	4.780 $^{a\gamma}$	2.523 $^{b\gamma}$	1.680 $^{c\alpha}$		
cis-12 C18:1	C	0.033 ^{a}	0.150 $^{b\alpha}$	0.110 $^{c\alpha}$	0.010	0.006	*cis*-9,*trans*-11 C18:2	C	0.000 ^{a}	0.021 $^{b\alpha}$	0.000 ^{a}	0.020	0.033
	S5	0.050 ^{a}	0.060 $^{a\beta}$	0.117 $^{b\alpha}$				S5	0.000 ^{a}	0.112 $^{b\beta}$	0.000 ^{a}		
	S9	0.040 ^{a}	0.040 $^{a\beta}$	0.073 $^{b\beta}$				S9	0.000 ^{a}	0.113 $^{b\beta}$	0.000 ^{a}		
cis-13 C18:1	C	0.040 $^{a\alpha}$	0.070 $^{b\alpha}$	0.050 $^{a\alpha}$	0.013	0.046	*trans*-10,*cis*-12 C18:2	C	0.000	0.000 $^{\alpha}$	0.000	0.001	0.001
	S5	0.036 $^{a\alpha}$	0.040 $^{a\beta}$	0.073 $^{b\beta}$				S5	0.000 ^{a}	0.067 $^{b\beta}$	0.000 ^{a}		
	S9	0.080 $^{a\beta}$	0.036 $^{b\beta}$	0.053 $^{b\alpha}$				S9	0.000	0.000 $^{\alpha}$	0.000		
cis-15 C18:1	C	0.020 $^{\alpha}$	0.020	0.037	0.009	0.045	*cis*-9,*cis*-12,*cis*-15 C18:3	C	0.530 $^{a\alpha}$	0.393 $^{b\alpha}$	0.283 $^{c\alpha}$	0.006	0.044
	S5	0.036 $^{\alpha}$	0.036	0.033				S5	0.400 $^{a\beta}$	0.357 $^{b\beta}$	0.370 $^{b\beta}$		
	S9	0.053 $^{a\beta}$	0.023 ^{b}	0.020 ^{b}				S9	0.597 $^{a\gamma}$	0.337 $^{b\gamma}$	0.283 $^{c\alpha}$		
trans-9 C18:1	C	0.037	0.040	0.040	0.008	0.045	*cis*-9,*trans*-11,*cis*-15 C18:3	C	0.000	0.000	0.000 $^{\alpha}$	0.001	0.002
	S5	0.037	0.047	0.030				S5	0.000	0.000	0.000 $^{\alpha}$		
	S9	0.037	0.050	0.027				S9	0.000 ^{a}	0.000 ^{a}	0.056 $^{b\beta}$		
trans-10 C18:1	C	0.047	0.040	0.053	0.011	0.910	*trans*-11,*cis*-15 C18:2	C	0.000	0.000	0.000 $^{\alpha}$	0.001	0.004
	S5	0.060	0.063	0.057				S5	0.000	0.000	0.000 $^{\alpha}$		
	S9	0.043	0.050	0.047				S9	0.000 ^{a}	0.000 ^{a}	0.143 $^{b\beta}$		

α,β,γ Within a column, means with different Greek superscripts are significantly different ($P < 0.05$); a,b,c within a row, means with different Latin superscripts are significantly different ($P < 0.05$). C = control feed; S5 = treatment with 50 g/kg of SOP; S9 = treatment with 90 g/kg of SOP.

[1]Mean values with their standard errors (SEM); number of samples for each treatment at any time =3.

Microbial population profiling

DGGE analysis of PCR-amplified partial 16S rRNA genes was performed on total bacteria, *Butyrivibrio*, and methanogenic populations of RL incubated with the three diets. Microbial profiles obtained using universal primers for bacteria showed a complex band pattern in all samples (Figure 1A). UPGMA dendrogram separated samples incubated with S5 and S9 diets and collected at 24 h from all the other samples, with 82.8% similarity (Figure 1A). Within the cluster containing S5 and S9 and collected at 24 h two subclusters (86.2% similarity) were evident, based on the percentage of SOP (Figure 1A). Samples collected at 0 and 6 h formed a different group when compared with samples collected at 12 h and with control samples collected at 24 h, with a similarity of 87.6%. A similarity higher than 92% was found in RL samples inoculating C, S5 and S9 collected at 0 and 6 h (Figure 1A).

Figure 1 Cluster analysis based on unweighted pair group method with arithmetic mean of polymerase chain reaction denaturing gradient gel electrophoresis profiles showing the effect of C, S5 and S9 diets on total bacteria (A) and the *Butirivibrio* group (B) in rumen liquor collected at 0, 6, 12 and 24 h. C = control feed; S5 = treatment with 50 g/kg of stoned olive pomace; S9 = treatment with 90 g/kg of stoned olive pomace. Scale relates to percent similarity.

PCR-DGGE analysis of members of the *Butyrivibrio* group showed a less complex pattern than total bacteria (Figure 1B). Two main clusters were evident, separating all samples collected at 0 and 6 h from those collected at 12 and 24 h, with 77.0% similarity (Figure 1B). Subclusters once again reflected clearly the percentage of the amount of SOP added and the collection time (Figure 1B). Control samples collected at 0 and 6 h grouped differently from samples incubated with S5 and S9 diets (Figure 1B), with 81.2% similarity. Moreover, all samples collected at 12 h grouped separately from those collected at 24 h, with 85.6% similarity (Figure 1B). Along within the latter group, samples added with S5 and S9 diets grouped together, separating from control samples, with 90.6% similarity (Figure 1B).

PCR-DGGE profiles obtained from the analysis of methanogens did not show differences at any sampling time for all feeds (data not shown).

Sequence analysis of bacterial and *Butyrivibrio*-specific PCR-DGGE bands

PCR-DGGE bands exhibiting remarkable changes in response to SOP in total bacteria or in *Butyrivibrio* populations (bands 1, 5, 7, 8, 9, 11, 12, 17, 18, 20, 21, 22, 23 and 24) were excised, re-amplified and sequenced (Figure 2). Moreover, in order to gain more information on the composition of rumen bacterial community of sheep, ten bands obtained with primers F968/R1401 for total bacteria (bands 2, 3, 4, 6, 10, 13, 14, 15, 16 and 19) were selected and sequenced, even if their intensity was not affected by SOP (Figure 2A). Putative taxonomic identification of each band subjected to sequencing is reported in Table 4.

Figure 2 PCR-DGGE profiles of total bacterial community (A) and *Butyrivibrio* members (B) in rumen liquor inoculating C, S5 and S9 diets and collected at 0, 6, 12 and 24 h. C = control feed; S5 = treatment with 50 g/kg of stoned olive pomace; S9 = treatment with 90 g/kg of stoned olive pomace. Bands indicated by numbers were selected for sequencing.

Table 4 Identification of the selected polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) fragments

PCR-DGGE band	Nearest match (GenBank accession no.; % sequence similarity)	Taxonomic classification
Total bacterial community analysis		
1	*Pasteurella testudinis* (NR_042889; 90%)	Unclassified *Pasteurellaceae*
2	*Bergeriella denitrificans* (NR_040933; 99%)	*Bergeriella denitrificans*
3	*Bergeriella denitrificans* (NR_040933; 99%)	*Bergeriella denitrificans*
4	*Clostridium lavalense* (NR_044289; 93%)	Unclassified *Clostridiaceae*
5	*Neisseria weaveri* (NR_025902; 99%)	*Neisseria weaveri*
6	*Neisseria weaveri* (NR_025902; 98%)	*Neisseria weaveri*
7	*Neisseria weaveri (*NR_025902; 99%)	*Neisseria weaveri*
8	*Neisseria weaveri* (NR_025902; 98%)	*Neisseria weaveri*
9	*Neisseria flavescens* (KF030235; 100%)	*Neisseria flavescens*
10	*Clostridium citroniae* (NR_043681; 90%)	Unclassified *Clostridiaceae*
11	*Ruminobacter amylophilus* (NR_026450; 99%)	*Ruminobacter amylophilus*
12	*Neisseria flavescens* (KF030235; 100%)	*Neisseria flavescens*
13	*Neisseria flavescens* (KF030235; 100%)	*Neisseria flavescens*
14	*Neisseria weaveri* (NR_025902; 98%)	*Neisseria weaveri*
15	*Howardella ureilytica* (NR_044022; 94%)	Unclassified *Clostridiaceae*
16	*Roseburia faecis* (NR_042832; 90%)	Unclassified *Lachnospiraceae*
17	*Butyrivibrio hungatei* (NR_025525; 90%)	Unclassified *Lachnospiraceae*
18	*Butyrivibrio hungatei* (NR_025525; 93%)	Unclassified *Lachnospiraceae*
19	*Ruminococcus torques* (NR_036777; 90%)	Unclassified *Lachnospiraceae*
Butyrivibrio-specific analysis		
20	*Butyrivibrio proteoclasticus* (NR_102893; 92%)	Unclassified *Lachnospiraceae*
21	*Butyrivibrio proteoclasticus* (NR_102893; 98%)	*Butyrivibrio proteoclasticus*
22	*Butyrivibrio proteoclasticus* (NR_102893; 99%)	*Butyrivibrio proteoclasticus*
23	*Robinsoniella peoriensis* (NR_041882; 94%)	Unclassified *Lachnospiraceae*
24	*Eubacterium ruminantium* (NR_024661; 92%)	Unclassified *Lachnospiraceae*

Phylogenetic analysis of the nineteen sequences of the PCR-DGGE fragments obtained with primers F968/R1401 (total bacteria) and sequences from rumen bacteria of equivalent length retrieved from the GenBank database was performed. The results indicated that seven sequences (bands 4, 10, 15, 16, 17, 18 and 19) were related to known species of *Clostridiales* (Figure 3), ten sequences (bands 2, 3, 5, 6, 7, 8, 9, 12, 13 and 14) were related to *Neisseriales* and the remaining two sequences were related to *Pasteurellales* (band 1) and *Aeromonadales* (band 11) (Figure 3, Table 4).

Figure 3 Neighbour-joining tree built using all 16S rRNA sequences obtained from total bacteria PCR-DGGE gels and sequences of rumen bacteria of equivalent length, retrieved from the GenBank database. Sequences obtained in this study are shown in boldface. Bootstrap values >50% based on 1000 replications are indicated at the nodes. The 16S rRNA gene sequence of *Escherichia coli* (NR_024570) was selected as the outgroup.

The analysis of total bacterial PCR-DGGE profiles evidenced that seven bands, corresponding to *Neisseria weaveri* (bands 5, 7 and 8), *Ruminobacter amylophilus* (band 11), unclassified *Pasteurellaceae* (band 1) and *Lachnospiraceae* (bands 17 and 18) reduced their intensity at 24 h in samples receiving the S9 diet in comparison to controls, whereas one band, identified as *Neisseria flavescens* (band 9), increased in S9 samples at the same sampling time (Figure 2A). On the contrary, minor differences were observed in presence of S5 diet at 24 h in comparison to controls, since the only disappearance of band 12 (*Neisseria flavescens*), and the appearance of band 9 (*Neisseria flavescens*) were detected (Figure 2A).

A phylogenetic tree was also constructed with the five sequences obtained with the *Butyrivibrio*-specific primers F968/Bfib and other sequences of equivalent length, representative of bacterial species related to the *Lachnospiraceae* family. As shown in Figure 4, two sequences (bands 21 and 22) grouped with sequences representative of *Butyrivibrio proteoclasticus*, whereas three sequence (band 20, 23 and 24) displayed a very low level of similarity with other known bacterial species belonging to the *Lachnospiraceae* family.

Figure 4 Neighbour-joining tree built using all 16S rRNA sequences obtained from *Butirivibrio*-specific PCR-DGGE gels and sequences of rumen bacteria of equivalent length, retrieved from the GenBank database. Sequences obtained in this study are shown in boldface. Bootstrap values of >50% based on 1000 replications are indicated at the nodes. The 16S rRNA gene sequence of *Escherichia coli* (NR_024570) was selected as the outgroup.

PCR-DGGE profiles obtained using *Butyrivibrio*-specific primers showed weak changes in the *Butyrivibrio* community in relation to diets. In S5 and S9 samples bands 21 and 22, identified as *Butyrivibrio proteoclasticus*, reduced their intensity already at 12 h when compared to C (Figure 2B). Moreover, considering samples collected at 24 h, band 20, (unclassified *Lachnospiraceae*), showed a lower intensity than controls after incubation with S5 and S9 diets, whereas bands 23 and 24 (unclassified *Lachnospiraceae*) decreased slightly only in presence of S9 diet (Figure 2B).

Discussion

In literature it is well known that the inclusion of plant polyphenols in animal feed interferes with rumen metabolism, decreasing dietary protein degradation for a better rumen energy-

protein balance and reducing methanogenesis by means targeting specific group of microorganisms [1,5]. SOP, by-product of olive oil extraction containing a high proportion of polyphenols, has been proposed as supplement in ruminant feeding with the aim to improve the content of PUFA in dairy products deriving from ruminant livestock, contributing at the same time to the environmental sustainability of animal productions [7,10]. However, until now, there is a lack of knowledge on the effect of SOP on lipid metabolism and rumen microbial communities involved in fatty acid BH processes. This information is essential in order to optimize its employment in ruminant feeding.

According with a validate experimental design [1], our finding showed that the inclusion of SOP in feeds stimulated the production of volatile fatty acids, suggesting that microbial activity was modified by the presence of polyphenols in feeds: the highest increase of C3:0 in the fermented RL inoculating S5 and S9 can be related to a good level of amilolytic bacteria activity, while the constant production of C2:0 and the increase of *iso* C5, arising from microbial degradation of dietary aminoacids, can be an indication of a stimulated cellulolytic bacteria activity [11]. *Iso* C5 is the precursor of *iso* C15 and *iso* C17, which arise from the peculiar rumen methabolism of cellulolytic bacteria [11]. In our experiment *iso* C15 and *iso* C17 production was stimulated by SOP confirming that cellulolytic activity was not perturbed although literature demonstrated the antimicrobial activities of olive oil mill waste-waters against different groups of bacteria [12].

SOP supplementation in feeds did not contribute to protect double bond *cis*-9 from the saturation as demonstrated by BH of OA and RA that decreased during the fermentation period. A decrease of OA isomerisation to other *trans* C18:1 isomers could be hypothesized because not significant variations in the concentration of these monoenes have been detected. The temporary RA accumulation at 12 h in RL fermented with S5 and S9 can be related to a negative feed-back effect caused by the VA accumulation in these fermenters. This hypothesis could be extended also to conjugated linolenic acid and vaccelenic acid, further precursor of VA from α-LNA biohydrogenation, which appeared only at the end of fermentation in fermenters containing the highest content of SOP. VA accumulation in RL fermented with S5 and S9 is closely related to a decrease in *Butyrivibrio proteoclasticum* growth as revealed by PCR-DGGE analysis, as following discussed. SOP did not contribute to preserve LA and α-LNA from isomerisation to their *cis*-9,*trans*-11 isomers, indicating that LA-Isomerase activity is not influenced by SOP inclusion in feeds. Moreover, the shift of LA and α-LNA biohydrogenation toward the *trans*-10 isomers falls is not enhanced. This trend agrees with several studies that demonstrated polyphenols do not favour the increase of *trans*-10 monoenes synthesis [1,5,7].

Cluster analysis of PCR-DGGE profiles obtained with universal primers for 16S rRNA gene clearly showed a shift in total bacterial community in presence of SOP-enriched diets, in comparison to controls. Buccioni et al. [1] evidenced that tannins, a class of polyphenols, were able to affect the FA composition of solid- and liquid-associated bacteria communities from the rumen of sheep, suggesting changes in their composition and/or activity in relation to the BH process. In our study, the effect of SOP on rumen bacterial communities seemed to depend on the level of its supplementation in the diet and on the incubation time. Indeed, after 24 h of incubation with 90 g/kg SOP some bands in PCR-DGGE profiles showed a decreased intensity. We thus hypothesize that the changes observed in PCR-DGGE banding pattern may reflect the reduced abundance of the most sensitive species of ruminal bacteria to the antimicrobial action of SOP. Our observation is in agreement with previous *in vitro* studies, underlining that polyphenols from different plants are able to reduce the activity and

the proliferation of different ruminal microorganisms [13]. The inhibitory effect exerted by this compounds has been explained by their ability to form complexes with the bacterial wall and to inactivate many extracellular enzymes secreted [14].

Until now few studies have been carried out on sheep rumen microbiota using PCR-DGGE analysis followed by sequencing and identification of the main bacterial groups. Here only eleven PCR-DGGE bands obtained from the total bacterial community analysis resulted highly related to the 16S rRNA gene of known species, whereas the other ones correspond to yet unclassified bacteria. This result is not surprisingly, since the use of different culture-independent methods has demonstrated that rumen microbiota is more diverse than previously hypothesized taking into account the number of cultivated species [3]. On the whole, the sequenced bands resulted related mainly to species belonging to the *Clostridiaceae* family and to the genus *Neisseria*. The first taxonomic group includes many cellulolytic and amylolytic species, often found in the rumen [3]. On the contrary, only a gram-negative carbohydrate-fermenting bacteria similar to *Neisseria* has been isolated from sheep rumen [15]. However, since the members of the *Neisseriaceae* family are mammalian commensals [16], their presence in the rumen is likely. In the analyzed samples, we also detected the presence of *Ruminobacter amylophilus*, a typical rumen bacteria that may occur in reasonably large number in high grain or high roughage diets [17].

The most interesting changes in PCR-DGGE profiles were observed after 24 h in RL inoculating S9 diet for the species *Neisseria flavescens, Neisseria weaveri, Ruminobacter amylophilus* and for members of the *Lachnospiraceae* and *Pasteurellaceae* families. Previous *in vitro* and *in vivo* studies have shown that some members of the *Lachnospiraceae* family, such as *Butyrivibrio* species, are the main known bacteria involved in rumen BH [18]. Nevertheless, analyzing the RL of dairy cows by T-RFLP and DGGE approaches [19], have recently suggested that other yet not known bacterial species may play a role in the BH process. Thus, according to these authors, the findings obtained until now from studies on pure cultures may be not able to explain the bacterial contribution to rumen BH *in vivo*, that appears more complex than previously thought. Our study strengthens this hypothesis, even if further research is need to clarify the potential role of the detected bacterial groups in FA metabolism of sheep rumen.

Previous *in vitro* experiments have shown that members of the *Butyrivibrio* group are able to biohydrogenate unsaturated FA more rapidly than other species. However, among this group, only *B. proteoclasticus* has been recognized to reduce C18:1 to C18:0 [4]. Since members of the *Butyrivibrio* group comprise only a minor part of ruminal bacteria [20] we performed a *Butyrivibrio*-specific PCR-DGGE analysis to investigate in detail the effect of SOP supplementation on this taxonomic group. Cluster analysis showed that both diets supplemented with SOP affected the composition of the *Butyrivibrio* population. Indeed, both at 12 h and at 24 h of incubation we observed a reduced intensity of specific PCR-DGGE bands. This result is consistent with previous *in vitro* observations that evidenced the sensitivity of some members of the *Butyrivibrio* group to polyphenol extracts obtained from tannin-rich plants [13,14]. Sequence analysis revealed that two bacterial group responding negatively to SOP after 12 h of incubation were closely related to *B. proteoclasticus* (levels of 16S rDNA similarity above 98.0%), that is the only cultured SA producer. Since a significant increase of VA was observed in relation to incubation time only in samples added with SOP, we hypotheses that *B. proteoclasticus*, and other species of *Butyrivibrio* here not identified, might play a role in the conversion of *trans* C18:1 to C18:0. Our data confirm the hypothesis formulated by Vasta et al. [5], who found a correlation between the reduced

abundance of *B. proteoclasticus* and the contemporary increase of VA in lamb rumen fluid, following the addiction of polyphenols from quebracho tannins to the diet. Thus, our data suggest that SOP may decrease the hydrogenation of *trans* C18:1 and *trans* C18:2 intermediates by affecting negatively the growth of *B. proteoclasticus*.

The present study reports also some results on the effect of SOP on rumen archeal methanogens, in order to elucidate if addition of this type of feed to ruminant diet may cause shifts in the methanogenic community. Surprisingly, we found that SOP addition did not significantly affect methanogen diversity and relative abundance, independently of the time of sampling and of the dietary SOP level. Until now few studies have dealt with the effect of polyphenols on rumen methanogenic communities, even if a study carried out by Jeyanathan et al. [21] showed that archaeal communities remained relatively constant across different ruminant species and diets, differently from bacterial communities. Our data are in agreement with these findings, but further investigations are required to understand if SOP may affect total methanogens abundance, that has been suggested to be more important in determining methane emission rates than the composition of methanogenic community [22].

Conclusions

Supplementation of feeds with SOP inhibited in a dose dependent manner the rumen BH of C18 unsaturated FA, resulting in a decrease of SA concentration and in an increase of VA. In particular, changes in rumen fatty acid profile were associated with changes in the bacterial community, including bacteria responsible for VA hydrogenation to SA. Hence, an use of SOP aimed to produce meat or dairy products enriched in functional lipids could be hypothesized. Moreover, the use of SOP in animal feeding can represent a revaluation of a bio-waste from food chain, thus contributing to environmental sustainability.

Methods

Feed composition

Feeds used as substrate of the fermentation were: a control diet (C) in which the SOP was not included and other two diets (S5 and S9) in which the integration with SOP was respectively of 50 g/kg on DM and 90 g/kg on DM. The amount of SOP used in this experiment was chosen with the criteria of the practicality under farm conditions. The diets were formulated to be isoproteic and isoenergetic. The ingredients and chemical composition of feeds are showed in Table 5. SOP was obtained after mechanical extraction of virgin olive oil using the following operative conditions [10]: the olives were stoned and malaxed for 40 min at 25°C and the oil extraction was performed using an RCM Rapanelli three phases decanter mod. 400 eco. After storage at room temperature for 36 hours, stoned olive cake was dried using a fluid bed dryer; the initial temperature of the drying air flow was 120°C and the maximum temperature of olive cake during the drying process was 45°C. The dried stoned olive cake was stored at room temperature. The proximate composition (according to A.O.A.C procedures, 1990) of SOP was: DM (873.80 g/kg), crude protein (118.31 g/kg on DM), neutral detergent fibre (490.51 g/kg on DM), acid detergent fibre (347.40 g/kg on DM), acid detergent lignin (85.61 g/kg on DM) and 63.43 g/kg on DM of crude fat in which the main FA contained were C16:0 (12.81 g/100 g of total FA), *cis-9* C18:1 (76.43 g/100 g of total FA) and *cis-9,cis-12* C18:2 (6.82 g/100 g of total FA). Polyphenols composition of SOP was determined according to Servili et al. [9]: 3,4-dihydroxyphenolethanol (1.16 g/kg DM), 4-

hydroxyphenolethanol (0.11 g/kg DM); p-cumaric acid (0.04 g/kg DM), verbascoside (1.33 g/kg DM), 2-(3,4-hydroxyphenyl)ethyl(3S,4E)-4-formyl-3-(2-oxoethyl)hex-4-enoate (1.16 g/kg DM). Total polyphenols content in SOP was 3.80 g/kg DM.

Table 5 Composition of feeds used as substrate of the fermentation and main fatty acids (FA) in rumen liquor (RL) at the start of fermentation

Feed composition	C	S5	S9
Ingredients (g/kg DM)			
Grass hay	103.45	103.45	98.04
Wheat straw	103.45	103.45	98.04
Mais meal	545.52	510.00	504.80
Soybean meal	42.76	42.76	40.52
Wheat bran	33.10	33.10	31.37
Bean flakes	20.69	20.69	19.61
Soybean flakes	12.41	12.41	11.76
Horsebean flakes	11.03	11.03	10.46
Barley	109.66	95.17	78.43
Stoned olive oli cake	---	50.00	90.00
Maize germ meal	17.93	17.93	16.99
Chemical composition (g/kg DM)			
Crude protein (6.25 x N)	115.91	116.23	116.40
Crude fat	23.42	24.51	25.63
Neutral detergent fiber	366.00	379.40	391.81
Acid detergent fiber	194.73	205.63	215.82
Lignin	81.64	81.81	82.53
Ash	58.76	61.25	63.56
Non protein nitrogen	73.92	83.83	89 · 62
Soluble protein	21.37	36.50	46.30
Neutral detergent insoluble protein	21.61	48.31	68.91
Acid detergent insoluble protein	9.60	19.80	27.73
Main fatty acids in RL at the start of fermentation (g/100 g of total FA)			
C16:0	18.05	17.23	16.33
C18:0	1.90	1.79	1.79
cis-9 C18:1	23.92	25.19	26.81
cis-9,*cis*-12 C18:2	52.96	52.98	52.38
cis-9,*cis*-12,*cis*-15 C18:3	2.67	2.44	2.32

C = control feed; S5 = treatment with 50 g/kg of stoned olive pomace (SOP); S9 = treatment with 90 g/kg of SOP.

In vitro incubation with sheep ruminal fluid

The *in vitro* incubation was performed according to Tedeschi et al. [23] with several modification: Four sheep, conditioned with a basal diet formulated to shape rumen microflora and composed by grass hay (770 g/kg DM), soybean meal (55 g/kg DM), barley meal (175 g/kg DM), were used to provide rumen contents. Animals had continuous access to water and mineral blocks. After 4 weeks of adaptation period, about 1 litre of rumen contents was collected from each sheep using a rumen fluid sampling pump on the same day before the morning meal. The handling of the animals were according to Institutional Animal Care and Use Committee of Florence University (IACUC, 2004). The RL was immediately mixed with CO_2 to avoid O_2 contamination and transferred to the laboratory in a thermostatic box (39°C) under anaerobic condition. The RL was than filtered through four layers of cheese cloth into a

flask under a continuous flux of CO_2. An aliquot of the RL was buffered (1:3, v/v) by adding an artificial saliva solution [24]. Feed (2 g DM) were incubated in triplicate with 200 ml of inoculum. The incubator consisted of a thermostatic chamber (39°C) equipped with twenty-seven 300 ml glass fermentation vessels provided with two inlets (one to release gas through a valve and one for the pH probe) and connected to an electronic pressure transducer (pre-set at 65 kPa) and to an electronic gas valve. When the inside gas pressure reached the pre-set value, the valve was opened releasing about 2 ml of gas. The fermentation pattern was monitored by a PC software (Labview 5.0, National Instr., Austin, TX). Each vessel, containing substrate inoculated with rumen fluid saturated with CO_2 to guarantee the anaerobic condition, was continuously stirred. Samples of RL were collected at 6, 12 and 24 h of incubation. According to Buccioni et al. [1] three vessels per treatment were used for rumen microbial characterization and FA analysis, as described below. Three aliquots of 1 ml of RL for each diet per sampling time combination were stored at -80°C until DNA extraction was performed.

Samples of RL (200 ml) immediately after the addition of buffer solution and before feed inoculation (as blank to control the quality) and samples of RL (200 ml) inoculated with feeds, at the start of fermentation (t = 0 min), were collected in triplicates to be analysed for FA profile. The fat content of RL blank was very low (0.01 g/l), as a consequence of the procedure adopted for the preparation of the inoculum; hence, the initial contribution of RL to FA composition of inoculum was negligible (data not shown). Table 5 shows the FA composition of RL inoculating the three diets at the beginning of fermentation. In the feeds the concentration of oleic acid (*cis*-9 C18:1, AO) increased according to the percentage of SOP inclusion in the diet.

Feeds proximate analysis

Samples of feeds were oven dried at 60°C for 24 h. The dry samples were analyzed for crude protein, ash and crude fat according to the 954.01, 954.05 and 920.39 procedures of AOAC (1990), respectively. Neutral detergent fiber, acid detergent fiber and acid detergent lignin were determined by using sequential analysis, with sodium sulfite, with heat stable amylase, and expressed inclusive of residual ash. The carbohydrate and protein differently degradable fractions (non protein nitrogen; soluble protein; neutral detergent insoluble protein; acid detergent insoluble protein) were estimated according to the Cornell Net Carbohydrates and Protein System CNCPS [25].

Rumen fatty acid analysis

To determine the FA, each sample (about 150 mg) was extracted according to Folch method [26] without drying the final solution containing the lipid extract which was directly methylated using a combination of methods according to Buccioni et al. [1] with the aim to avoid volatile fatty acid (VFA) loss. The first step consisted of an alkaline methylation with sodium methylate/methanol (1 ml of 0.5 M-Sodium Methoxide) to esterify glycerides. The second step involved an acid methylation with HCl/methanol (1.5 ml of 5% methanolic HCl, 10 min at 50°C) as catalyst to esterify NEFA. Fatty acid methylesters (FAME) were extracted using n-hexane with C9:0 and C23:0 methyl ester (Sigma Chemical Co., St. Louis, MO) as internal standards for quantification, and maintained in vials with hermetic closure to avoid the loss of volatile components. FAME were separated and identified by gaschromatography on a GC equipped with a capillary column (CP-select CB for FAME Varian, Middelburg, The Nederlands: 100 m × 0.25 mm i.d; film thickness 0.20 μm), according to Buccioni et al.

[27]. The injector and flame ionization detector temperatures were 270°C and 300°C, respectively. The programmed temperature was 40°C for 4 min, increased to 120°C at a rate of 10°C/min, maintained at 120°C for 1 min, increased to 180°C at a rate of 5°C/min, maintained at 180°C for 18 min, increased to 200°C at a rate of 2°C/min, maintained at 200°C for 1 min, increased to 230°C at a rate of 2°C/min and maintained at this last temperature for 19 min. The split ratio was 1:100 and helium was the carrier gas with a flux of 1 ml/min. Standard mix (47792 Supelco, Chemical Co., St. Louis, MO) and published isomeric profiles [28] were used to identify the α-linolenic acid (α-LNA) isomers. Two bacterial acid methyl ester mix (47080-U Supelco, Chemical Co., St. Louis, MO; GLC110, Matreya, Pleasant Gap, PA) and individual standard for methyl ester of *iso* C14:0, *anteiso* C14:0, *iso* C15:0 and *anteiso* C17:0 (21-1211-11, 21-1210-11, 21-1312-11 and 21-1415-11, Larodan Malmo, SW) were used to identify branched FA profile. Inter and intra-assay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Boureau of Reference, Bruxelles, Belgium) and detection threshold of FA was 0.01 g/100 g of FA. All FA composition results are expressed as g/100 g of FA.

DNA extraction from rumen microbial samples

Genomic DNA was extracted from 1 ml of rumen microbial suspension using the Fast DNA SPIN kit for soil (Qbiogene, Carlsbad, CA, USA) with some modifications. Briefly, each sample was thawed and transferred to a 15 ml tube containing 4.5 ml of lysis buffer (500 mM-NaCl; 50 mM-Tris-HCl, pH 8.0; 50 mM-EDTA and 4% SDS) and incubated for 15 min at 70°C with gentle shaking by hand every 5 min. After centrifuging at 200 × *g* at 4°C for 5 min, 1 ml of the supernatant was transferred to a 2 ml centrifuge tube and centrifuged at 14,600 × *g* at 4°C for 5 min. The supernatant was removed and the pellet was dissolved in 978 µl of buffer sodium phosphate and 122 µl of MT buffer (both solutions are supplied by the Fast DNA SPIN kit for soil). Each sample was homogenized with a FastPrep cell disrupter instrument (Bio101, ThermoSavant, Qbiogene, Carlsbad, CA, USA) for 2 × 40 s at speed 6.0 and then processed according to the manufacturer's guidelines. This combination of methods was used to maximize the recovery of DNA from ruminal digesta. DNA was eluted in sterile water and its integrity was verified by agarose gel electrophoresis. The amount and the purity of DNA was measured at 260 and 280 nm using ND-1000 Spectrophotometer (NanoDrop Technologies, Labtech, Ringmer, UK).

PCR-DGGE analysis of the total bacterial community, *Butyrivibrio* and methanogens groups

Individual total DNA extracted from rumen samples was diluted to a concentration of 5 ng/µl and 2 µl of diluted DNA was used as template in PCR reactions. Amplification of the V6-V8 region of the 16S rRNA gene was carried out with the primer pair F968GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and R1401 (5'-CGG TGT GTA CAA GAC CC-3') [29] for total eubacterial PCR (fragment size ~470 bp) and with F968GC and Bfib (5'-TTC GGG CAT TYC CRA CT-3') [30] for *Butyrivibrio* group-specific PCR (fragment size ~470 bp). In addition, primer pair F787GC (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAT TAG ATA CCC SBG TAG TCC-3') and R1240 (5'-CCA TTG TAG CCC GCG T-3') [31], targeting the V5-V7 region of the 16S rRNA gene (~480 bp), was used for ruminal methanogenic community profiling. Reactions were carried out using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hertfordshire, UK) in 25 µl volumes containing 1X PCR buffer (67 mM-Tris-HCl, pH 8.8; 1.66 mM-$(NH_4)_2SO_4$; 0.1% Tween-20), 1.5 mM-$MgCl_2$,

250 μM-deoxynucleotide triphosphates (dNTPs), 400 nM each primer, and 1U of Polytaq (Polymed, Florence, Italy). Amplifications were performed under the following conditions: an initial denaturation of 94°C for 5 min followed by 35 cycles of 94°C for 20 s, 56°C (total bacteria and *Butyrivibrio* group) or 55°C (methanogens) for 30 s and 72°C for 45 s, and a final extension of 72°C for 10 min. After PCR, amplified products were verified by agarose gel electrophoresis. Subsequently, in order to perform polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis, amplicons were loaded on a 6% polyacrylamide gel (acrylamide/bis 37.5:1), with a 50-60% (total bacteria and *Butyrivibrio* group) or 50-65% (methanogens) denaturing gradient (100% denaturant consisting of 40% v/v deionized formamide, 7 M-urea) and electrophoresis was performed in a Phor-U system (Ingeny International, Goes, NL). The gel was run for 17 h at 60°C and 75 V and, after electrophoresis, stained with SYBR® Gold (Molecular Probes, Eugene, OR) and scanned using ChemiDoc XRS (Bio-Rad Laboratories, Hertfordshire, UK).

The PCR-DGGE banding patterns obtained were analyzed using the software package GelCompar II Software v 4.6 (Applied Maths, Saint-Martens-Latem, Belgium). Normalization of bands within and between gels was performed by defining an active reference system. To summarize the species number of rumen bacterial communities, each band was considered as corresponding to a single microbial specie. Bands with a minimum area below 1% were discarded.

The banding patterns of PCR-DGGEs were further analyzed by hierarchical cluster analysis based on position and presence/absence of bands in the different profiles. The resultant binary matrices were translated into distance matrices using the Dice similarity coefficient and utilized to construct dendrogram using the unweighted pair group method using arithmetic average (UPGMA) algorithm.

Sequence analysis of PCR-DGGE fragments

A total of 30 bands were excised from the DGGE gels and were placed in 20 μl distilled water. The PCR products were eluted through freezing and thawing according to Throbäck et al. [32] and reamplified using the F968/R1401, F968/Bfib or F787/1240R primers without GC clamp, as described above. The fresh PCR products were then sequenced by dideoxy chain termination method at BMR Genomics sequencing service (BMR Genomics srl, Padova, Italy). Sequence chromatograms were visualized using the computer software Finch TV (ver. 1.4.0, Geospiza, Seattle, USA). Nucleotide sequences were compared against all sequences in GenBank release using BLASTN program [33] in order to identify the microorganisms corresponding to each selected band. Taxonomic identification was achieved by using different sequence similarity thresholds: a similarity ≥97% for a species level identification and 95%, 90%, 85%, 80% and 75% for assignment at the genus, family, order, class and phylum level, respectively [34].

For phylogenetic analysis sequences were aligned together with other sequences of equivalent length retrieved from the GenBank database, using the ClustalX 2.0.11 multiple sequence alignment software [35]. Distance calculation was performed according to Jukes and Cantor [36] followed by phylogenetic tree construction using the neighbor-joining algorithm [37] by means of TREECON 1.3b [38]. The robustness of each node was evaluated by bootstrap analysis with 1000 replicates.

Statistical analysis of fatty acids data

Data of FA concentration were analysed according to Buccioni et al. [1] and processed by General Linear Model of SAS [39] using the following linear model with fixed factors: diet and incubation time as well as their interaction.

$$y_{ij} = \mu + F_1 + T_j + F_i \times T_j + e_{ij}$$

where y_{ij} is the observation; μ is the overall mean; F_i the feed (i =1 to 3); Tj the incubation time (j =1 to 3); $F_i \times T_j$ the interaction between feed and incubation time and e_{ij} the residual error. Multiple comparisons of means were made using Tukey's adjustment. Main effect and differences were considered significant when $P < 0.05$.

Availability of supporting data

Nucleotide sequences from this study have been deposited in the GenBank database. Those from DGGE bands obtained with universal primer pair F968GC /R1401 targeting bacterial 16SrRNA gene have been deposited in the GenBank database under the accession numbers KF976364–KF976382. Those from DGGE bands obtained with primer pair F968GC/Bfib specific for the *Butyrivibrio* group have been deposited in GenBank under accession numbers KF976383-KF976387.

Band matching tables of Bacteria and *Butyrivibrio* DGGE profiles according to diet and time of sampling have been deposited to LabArchives, LLC (http://www.labarchives.com/) DOI: 10.6070/H4HH6H16.

Abbreviations

α-LNA, α-linolenic acid; BH, Biohydrogenation; CALNA, Conjugated linolenic acid; CLA, Conjugated linoleic acids; FA, Fatty acids; FAME, Fatty acid methylesters; LA, Linoleic acid; OA, Oleic acid; PCR-DGGE, Polymerase chain reaction denaturing gradient gel electrophoresis; RA, Rumenic acid; RL, Rumen liquor; SA, Stearic acid; SOP, Stoned olive pomace; VA, Vaccenic acid; VFA, Volatile fatty acids; VLA, Vaccelenic acid

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GP, AB and CV conceived the study; AB and SM performed fatty acid analysis; GP and RP performed DGGE experiments and analysis; GP, AB, RP, LG and CV prepared tables and figures; SM, MM, SR, AM, MP and MS gave technical support and conceptual advice. All authors discussed the results and implications and commented on the manuscript at all stages. All authors read and approved the final manuscript.

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References

1. Buccioni A, Minieri S, Rapaccini S, Antongiovanni M, Mele M: **Effect of chestnut and quebracho tannins on fatty acid profile in rumen liquid- and solid-associated bacteria: an in vitro study.** *Animal* 2011, **5:**1521–1530.

2. Griinari JM, Bauman DE: **Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants**. In *Advances in Conjugated Linoleic Acid Research.* Edited by Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW, Nelson GJ. Champaign: AOCS Press; 1999:180–200.

3. Kong YH, Teather R, Forster R: **Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages.** *FEMS Microbiol Ecol* 2010, **74:**612–622.

4. Wallace RJ, Chaudhary LC, McKain N, Mcewan NR, Richardson AJ, Vercoe PE, Walker ND, Paillard D: ***Clostridium proteoclasticum*: a ruminal bacterium that forms stearic acid from linoleic acid.** *FEMS Microbiol Lett* 2006, **265:**195–201.

5. Vasta V, Yanez-Ruiz DR, Mele M, Serra A, Luciano G, Lanza M, Biondi L, Priolo A: **Bacterial and protozoal communities and fatty acid profile in the rumen of sheep fed a diet containing added tannins.** *Appl Environ Microbiol* 2010, **76:**2549–2555.

6. Rochford S, Parker AJ, Dunshea FR: **Plant bioactives for ruminant health and productivity.** *Phytochemistry* 2008, **69:**299–322.

7. Luciano G, Pauselli M, Servili M, Mourvaki E, Serra A, Monahan FJ, Lanza M, Priolo A, Zinnai A, Mele M: **Dietary olive cake reduces the oxidation of lipids, including cholesterol, in lamb meat enriched in polyunsaturated fatty acids.** *Meat Sci* 2013, **93:**703–714.

8. Molina Alcaide E, Nefzaoui A: **Recycling of olive oil by-products: possibilities of utilization in animal nutrition.** *Int Biodet Biodeg* 1996, **38:**227–235.

9. Servili M, Esposto S, Fabiani R, Urbani S, Taticchi A, Mariucci F, Selvaggini R, Montedoro GF: **Phenolic compounds in olive oil: antioxidant, health and sensoryactivities according to their chemical structures.** *Inflammopharmacology* 2009, **17:**1–9.

10. Vargas-Bello Perez E, Vera RR, Auguillar C, Lira R, Peña I, Fernández J: **Feeding olive cake to ewes improves fatty acid profile of milk and cheese.** *Anim Feed Sci Technol* 2013, **184:**94–99.

11. Vlaeminck B, Fievez V, Tamminga S, Dewhurst RJ, van Vuuren A, De Brabander D, Demeyer D: **Milk odd- and branched-chain fatty acids in relation to the rumen fermentation pattern.** *J Dairy Sci* 2006, **89:**3954–3964.

12. Ramos-Cormenzana A, Juárez-Jiménez B, Garcia-Pareja MP: **Antimicrobial activity of olive mill waste-waters (alpechin) and biotransformed olive oil mill wastewater.** *Int Biodeter Biodegrad* 1996, **38:**283–290.

13. Min BR, Barry TN, Attwood GT, Mcnabb WC: **The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review.** *Anim Feed Sci Technol* 2003, **106:**3–19.

14. Jones GA, Mcallister TA, Muir AD, Cheng KJ: **Effects of sainfoin (*Onobrychis-viciifolia* Scop) condensed tannins on growth and proteolysis by 4 strains of ruminal bacteria.** *Appl Environ Microbiol* 1994, **60:**1374–1378.

15. Elsden SR, Volcani BE, Gilchrist FMC, Lewis D: **Properties of a fatty acid forming organism isolated from the rumen of sheep.** *J Bacteriol* 1956, **72:**681–689.

16. Sørum H, Sunde M: **Resistance to antibiotics in the normal flora of animals.** *Vet Res* 2001, **32:**227–241.

17. Caldwell DR, Bryant MP: **Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria.** *Appl Microbiol* 1966, **14:**794–801.

18. Boeckaert C, Vlaeminck B, Fievez V, Maignien L, Dijkstra J, Boon N: **Accumulation of trans C-18:1 fatty acids in the rumen after dietary algal supplementation is associated with changes in the *Butyrivibrio* community.** *Appl Environ Microbiol* 2008, **74:**6923–6930.

19. Huws SA, Kim EJ, Lee MRF, Scott MB, Tweed JKS, Pinloche E, Wallace RJ, Scollan ND: **As yet uncultured bacteria phylogenetically classified as *Prevotella*, Lachnospiraceae incertae sedis and unclassified Bacteroidales, Clostridiales and Ruminococcaceae may play a predominant role in ruminal biohydrogenation.** *Environ Microbiol* 2011, **13:**1500–1512.

20. Stevenson DM, Weimer PJ: **Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR.** *Appl Microbiol Biotechnol* 2007, **75:**165–174.

21. Jeyanathan J, Kirs M, Ronimus RS, Hoskin SO, Janssen PH: **Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets.** *FEMS Microbiol Ecol* 2011, **76:**311–326.

22. Lillis L, Boots B, Kenny DA, Petrie K, Boland TM, Clipson N, Doyle EM: **The effect of dietary concentrate and soya oil inclusion on microbial diversity in the rumen of cattle.** *J Appl Microbiol* 2011, **111:**1426–1435.

23. Tedeschi LO, Konohoff PJ, Karges K, Gibson ML: **Effects of chemical composition variation on the dynamics of ruminal fermentation and biological value of corn milling (co) products.** *J Dairy Sci* 2009, **92:**401–413.

24. McDougall EI: **Studies on ruminant saliva. I. The composition and output of sheep's saliva.** *Biochem* 1948, **43:**99–109.

25. Licitra G, Hernandez TM, VanSoest PJ: **Standardization of procedures for nitrogen fractionation of ruminant feeds.** *Anim Feed Sci Technol* 1996, **57:**347–358.

26. Folch J, Lees M, Sloane-Stanely GH: **A simple method for the isolation and purification of total lipids from animal tissues.** *J Clin Microbiol* 1957, **226:**497–507.

27. Buccioni A, Rapaccini S, Antongiovanni M, Minieri S, Conte G, Mele M: **Conjugated linoleic acid and C18:1 isomers content in milk fat of sheep and their transfer to Pecorino Toscano cheese.** *Int Dairy J* 2010, **20:**190–194.

28. Destaillats F, Trottier JP, Galvez JMG, Angers P: **Analysis of alpha-linolenic acid biohydrogenation intermediates in milk fat with emphasis on conjugated linolenic acids.** *J Dairy Sci* 2005, **88:**3231–3239.

29. Nubel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W, Backhaus H: **Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis.** *J Bacteriol* 1996, **178:**5636–5643.

30. Kim EJ, Huws SA, Lee MRF, Wood JD, Muetzel SM, Wallace RJ, Scollan ND: **Fish oil increases the duodenal flow of long chain polyunsaturated fatty acids and trans-11 18:1 and decreases 18:0 in steers via changes in the rumen bacterial community.** *J Nutr* 2008, **138:**889–896.

31. Baker GC, Smith JJ, Cowan DA: **Review and re-analysis of domain-specific 16S primers.** *J Microbiol Methods* 2003, **55:**541–555.

32. Throback IN, Enwall K, Jarvis A, Hallin S: **Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE.** *FEMS Microbiol Ecol* 2004, **49:**401–417.

33. Zhang Z, Schwartz S, Wagner L, Miller W: **A greedy algorithm for aligning DNA sequences.** *J Comput Biol* 2000, **7:**203–214.

34. Webster NS, Taylor MW, Behnam F, Lucker S, Rattei T, Whalan S, Horn M, Wagner M: **Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts.** *Environ Microbiol* 2010, **12:**2070–2082.

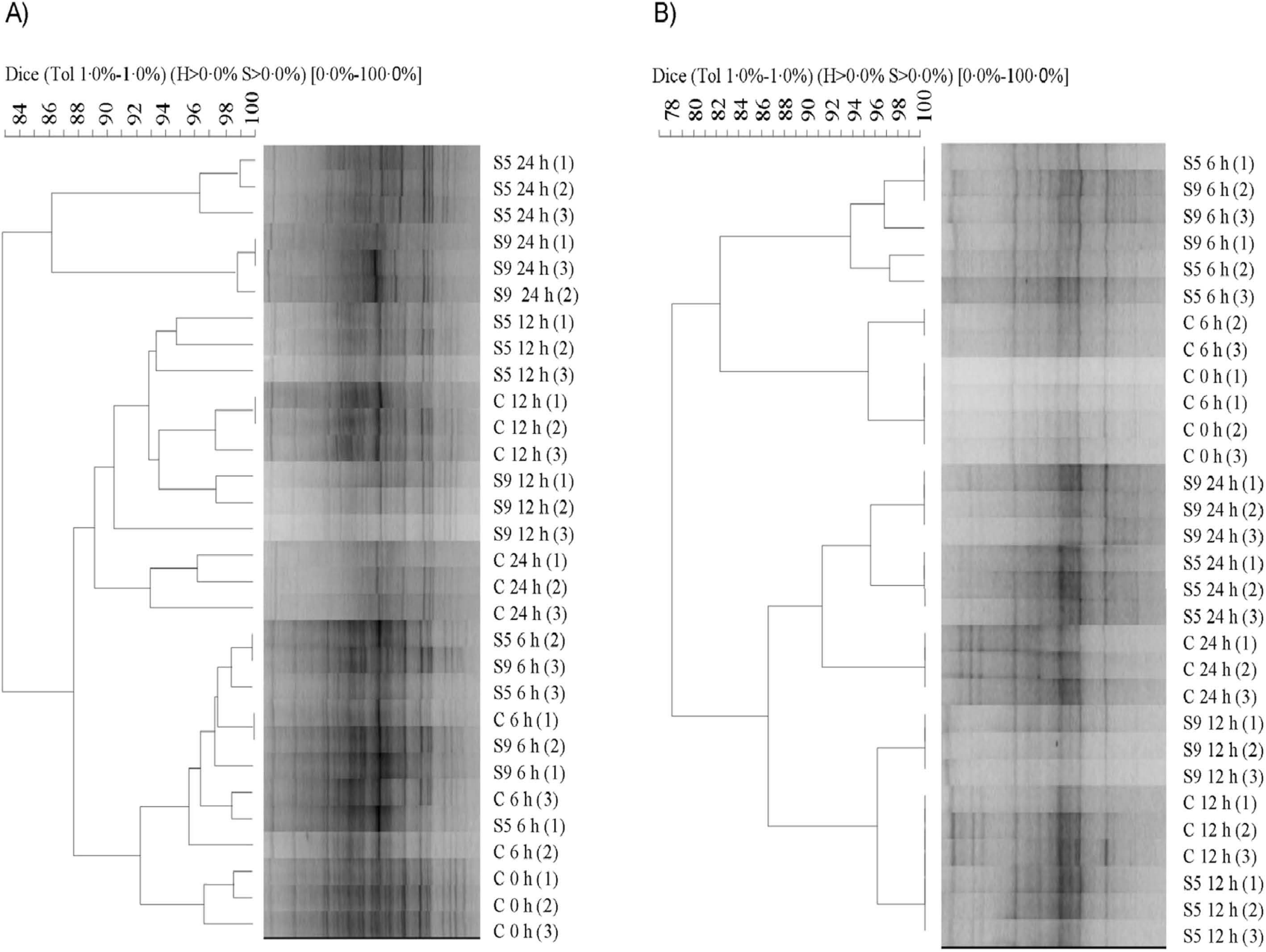
35. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettingan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG: **Clustal W and Clustal X version 2.0.** *Bioinformatics* 2007, **23:**2947–2948.

36. Jukes TH, Cantor CR: **Evolution of protein molecules**. In *Mammalian Protein Metabolism.* Edited by Munro HN. New York: Academic Press; 1969:21–132.

37. Saitou N, Nei M: **The neighbor-joining method - a new method for reconstructing phylogenetic trees.** *Mol Biol Evol* 1987, **4:**406–425.

38. Van de Peer Y, De Wachter R: **Treecon for Windows - a software package for the construction and drawing of evolutionary trees for the Microsoft Windows Environment.** *Comput Appl Biosci* 1994, **10:**569–570.

39. SAS Institute: *SAS User's Guide: Statistics. Version 8.0.* Cary: SAS Institute Inc; 1999.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S5 24 h (2)
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S9 24 h (1)
S9 24 h (3)
S9 24 h (2)
S5 12 h (1)
S5 12 h (2)
S5 12 h (3)
C 12 h (1)
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C 12 h (3)
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S9 6 h (3)
S5 6 h (3)
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C 6 h (2)
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C 0 h (2)
C 0 h (3)
B)
Dice (Tol 1·0%-1·0%) (H>0·0% S>0·0%) [0·0%-100·0%]
78 80 82 84 86 88 90 92 94 96 98 100
S5 6 h (1)
S9 6 h (2)
S9 6 h (3)
S9 6 h (1)
S5 6 h (2)
S5 6 h (3)
C 6 h (2)
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C 0 h (1)
C 6 h (1)
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S9 24 h (1)
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C 24 h (1)
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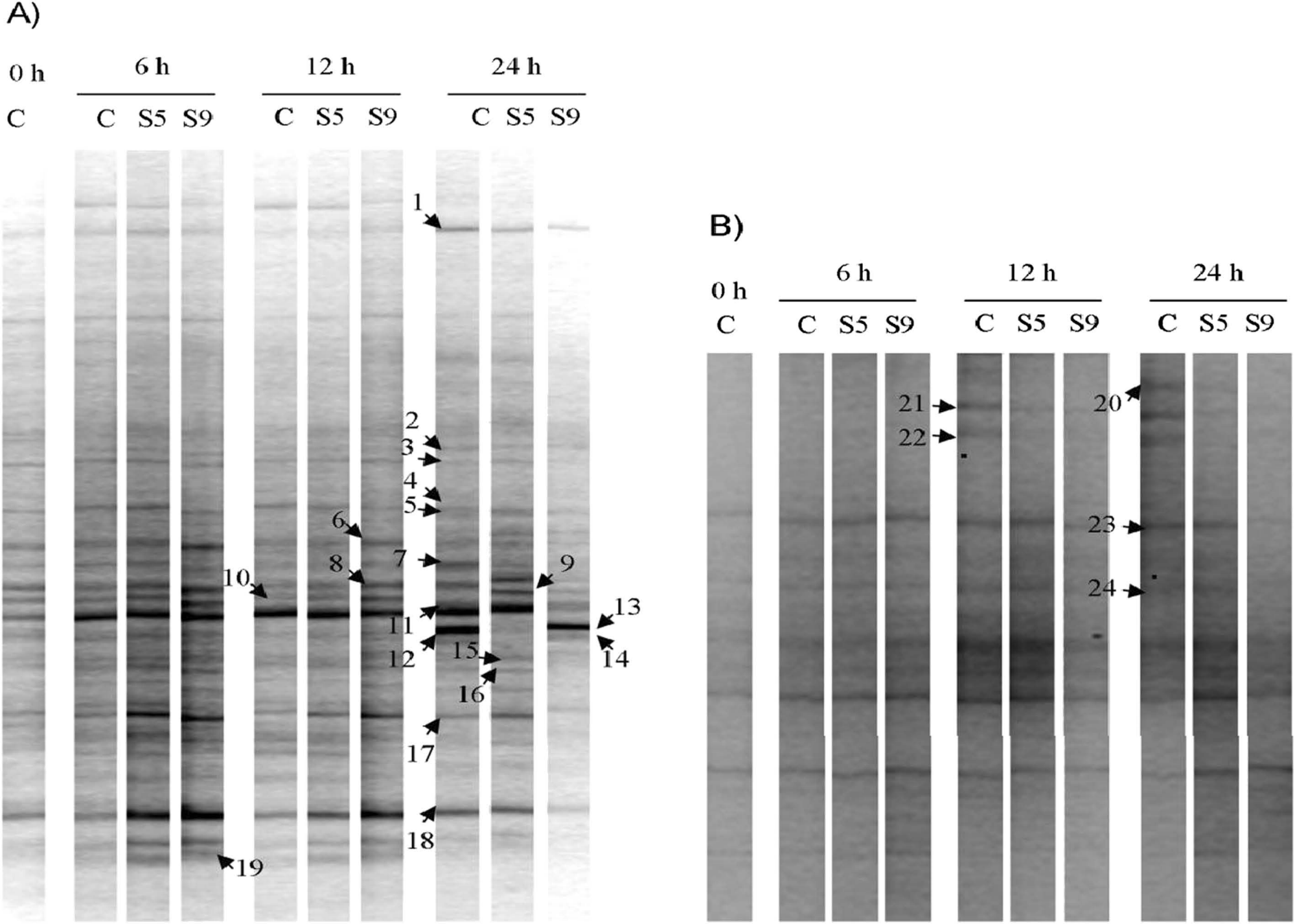
S5 12 h (3)


A)
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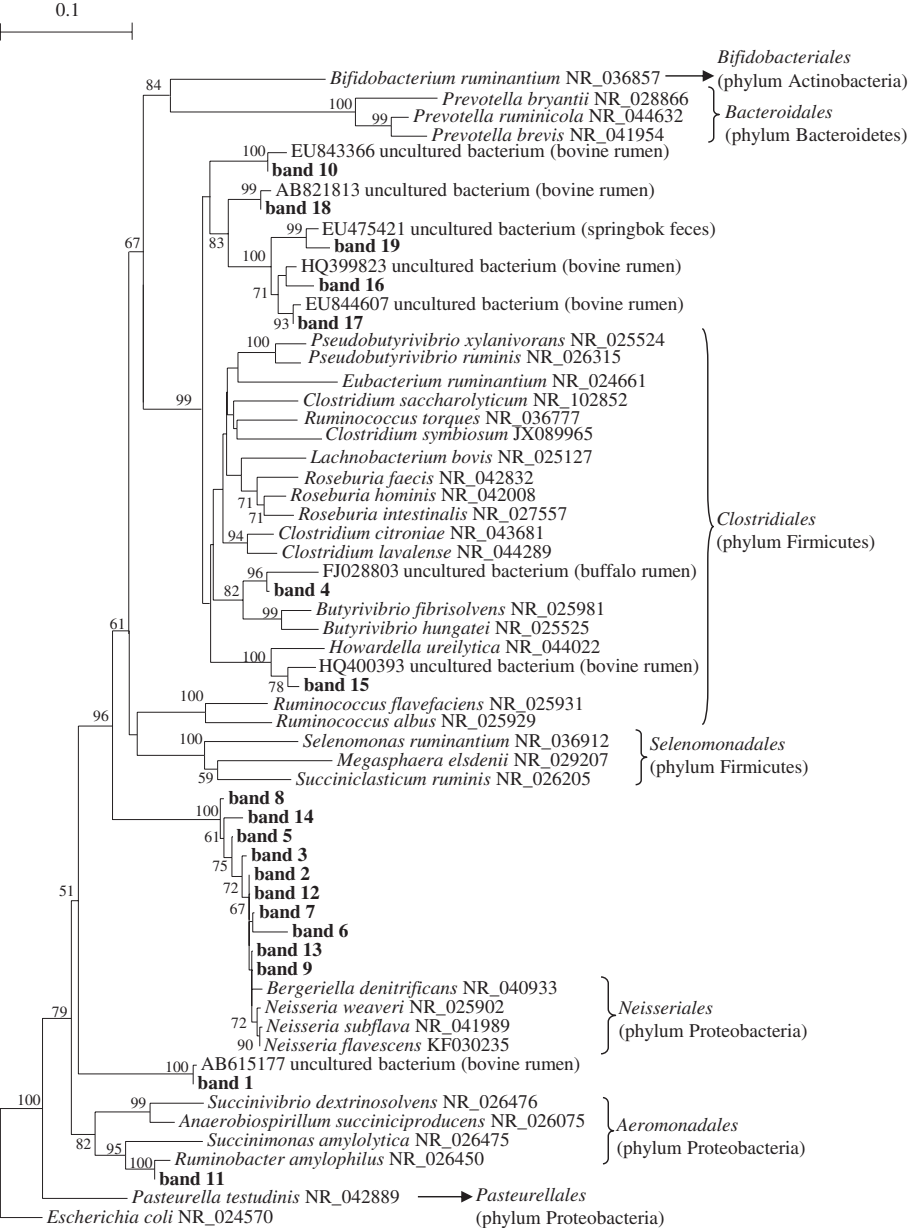
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Bifidobacteriales
(phylum Actinobacteria)
Bifidobacterium ruminantium NR_036857
Prevotella bryantii NR_028866
Prevotella ruminicola NR_044632
Prevotella brevis NR_041954
Bacteroidales
(phylum Bacteroidetes)
EU843366 uncultured bacterium (bovine rumen)
band 10
AB821813 uncultured bacterium (bovine rumen)
band 18
EU475421 uncultured bacterium (springbok feces)
band 19
HQ399823 uncultured bacterium (bovine rumen)
band 16
EU844607 uncultured bacterium (bovine rumen)
band 17
Pseudobutyrivibrio xylanivorans NR_025524
Pseudobutyrivibrio ruminis NR_026315
Eubacterium ruminantium NR_024661
Clostridium saccharolyticum NR_102852
Ruminococcus torques NR_036777
Clostridium symbiosum JX089965
Lachnobacterium bovis NR_025127
Roseburia faecis NR_042832
Roseburia hominis NR_042008
Roseburia intestinalis NR_027557
Clostridium citroniae NR_043681
Clostridium lavalense NR_044289
FJ028803 uncultured bacterium (buffalo rumen)
band 4
Butyrivibrio fibrisolvens NR_025981
Butyrivibrio hungatei NR_025525
Howardella ureilytica NR_044022
HQ400393 uncultured bacterium (bovine rumen)
band 15
Clostridiales
(phylum Firmicutes)
Ruminococcus flavefaciens NR_025931
Ruminococcus albus NR_025929
Selenomonas ruminantium NR_036912
Megasphaera elsdenii NR_029207
Succiniclasticum ruminis NR_026205
Selenomonadales
(phylum Firmicutes)
band 8
band 14
band 5
band 3
band 2
band 12
band 7
band 6
band 13
band 9
Bergeriella denitrificans NR_040933
Neisseria weaveri NR_025902
Neisseria subflava NR_041989
Neisseria flavescens KF030235
Neisseriales
(phylum Proteobacteria)
AB615177 uncultured bacterium (bovine rumen)
band 1
Succinivibrio dextrinosolvens NR_026476
Anaerobiospirillum succiniciproducens NR_026075
Succinimonas amylolytica NR_026475
Ruminobacter amylophilus NR_026450
band 11
Aeromonadales
(phylum Proteobacteria)
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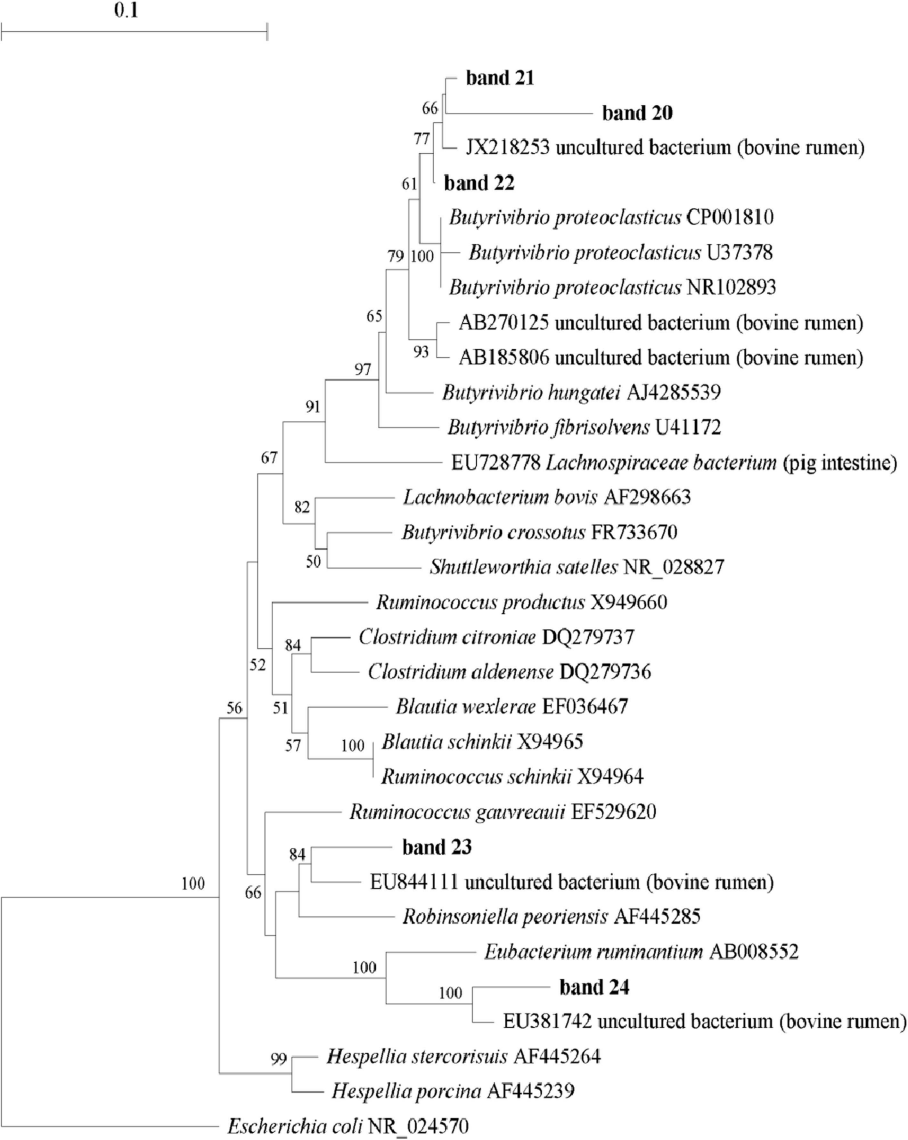
Escherichia coli NR_024570
Pasteurellales
(phylum Proteobacteria)


0.1
band 21
band 20
JX218253 uncultured bacterium (bovine rumen)
band 22
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AB270125 uncultured bacterium (bovine rumen)
AB185806 uncultured bacterium (bovine rumen)
Butyrivibrio hungatei AJ4285539
Butyrivibrio fibrisolvens U41172
EU728778 Lachnospiraceae bacterium (pig intestine)
Lachnobacterium bovis AF298663
Butyrivibrio crossotus FR733670
Shuttleworthia satelles NR_028827
Ruminococcus productus X949660
Clostridium citroniae DQ279737
Clostridium aldenense DQ279736
Blautia wexlerae EF036467
Blautia schinkii X94965
Ruminococcus schinkii X94964
Ruminococcus gauvreauii EF529620
band 23
EU844111 uncultured bacterium (bovine rumen)
Robinsoniella peoriensis AF445285
Eubacterium ruminantium AB008552
band 24
EU381742 uncultured bacterium (bovine rumen)
Hespellia stercorisuis AF445264
Hespellia porcina AF445239
Escherichia coli NR_024570