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Ruminal *Prevotella* spp. May Play an Important Role in the Conversion of Plant Lignans into Human Health Beneficial Antioxidants

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Introduction

Several human studies have revealed that ingestion of plant lignans, which are polyphenolic compounds classified as phytoestrogens, can decrease the incidence of menopausal symptoms, hormone-dependent cancers, cardiovascular diseases, osteoporosis and diabetes [1–5]. Flax (*Linum usitatissimum*) is the richest source of lignans [1], with secoisolariciresinol diglucoside (SDG) representing more than 95% of all flax lignans. Lignans are mainly found in the fibre portion of flax [6], thus resulting in higher concentration of lignans in hulls than seeds [7].

In monogastric animals, SDG is converted into secoisolariciresinol (SECO) under the action of intestinal glycosidases and the colonic microbiota convert SECO to the mammalian lignans enterolignans, which are strong antioxidants. Enterolactone (EL), the main mammalian enterolignan produced in the rumen, is transferred into physiological fluids, with potentially human health benefits with respect to menopausal symptoms, hormone-dependent cancers, cardiovascular diseases, osteoporosis and diabetes. However, no information exists to our knowledge on bacterial taxa that play a role in converting plant lignans into EL in ruminants. In order to investigate this, eight rumen cannulated cows were used in a double 4×4 Latin square design and fed with four treatments: control with no flax meal (FM), or 5%, 10% and 15% FM (on a dry matter basis). Concentration of EL in the rumen increased linearly with increasing FM inclusion. Total ruminal bacterial 16S rRNA concentration obtained using Q-PCR did not differ among treatments. PCR-T-RFLP based dendrograms revealed no global clustering based on diet indicating between animal variation. PCR-DGGE showed a clustering by diet effect within four cows that had similar basal ruminal microbiota. DNA extracted from bands present following feeding 15% FM and absent with no FM supplementation were sequenced and it showed that many genera, in particular *Prevotella* spp., contributed to the metabolism of lignans. A subsequent *in vitro* study using selected pure cultures of ruminal bacteria incubated with SDG indicated that 11 ruminal bacteria were able to convert SDG into secoisolariciresinol (SECO), with *Prevotella* spp. being the main converters. These data suggest that *Prevotella* spp. is one genus playing an important role in the conversion of plant lignans to human health beneficial antioxidants in the rumen.

Abstract

Secoisolariciresinol diglucoside (SDG), the most abundant lignan in flaxseed, is metabolized by the ruminal microbiota into enterolignans, which are strong antioxidants. Enterolactone (EL), the main mammalian enterolignan produced in the rumen, is transferred into physiological fluids, with potentially human health benefits with respect to menopausal symptoms, hormone-dependent cancers, cardiovascular diseases, osteoporosis and diabetes. However, no information exists to our knowledge on bacterial taxa that play a role in converting plant lignans into EL in ruminants. In order to investigate this, eight rumen cannulated cows were used in a double 4×4 Latin square design and fed with four treatments: control with no flax meal (FM), or 5%, 10% and 15% FM (on a dry matter basis). Concentration of EL in the rumen increased linearly with increasing FM inclusion. Total ruminal bacterial 16S rRNA concentration obtained using Q-PCR did not differ among treatments. PCR-T-RFLP based dendrograms revealed no global clustering based on diet indicating between animal variation. PCR-DGGE showed a clustering by diet effect within four cows that had similar basal ruminal microbiota. DNA extracted from bands present following feeding 15% FM and absent with no FM supplementation were sequenced and it showed that many genera, in particular *Prevotella* spp., contributed to the metabolism of lignans. A subsequent *in vitro* study using selected pure cultures of ruminal bacteria incubated with SDG indicated that 11 ruminal bacteria were able to convert SDG into secoisolariciresinol (SECO), with *Prevotella* spp. being the main converters. These data suggest that *Prevotella* spp. is one genus playing an important role in the conversion of plant lignans to human health beneficial antioxidants in the rumen.
EL has shown antioxidant activity [2], high levels of EL in milk may prevent oxidation [22] and increase shelf life of milk and dairy products.

The rumen microbiota responsible for the conversion of flax lignan into mammalian lignans is nonetheless unknown. Therefore, the aims of this study were to evaluate the effects of inclusion of FM in the diet of dairy cows on the ruminal microbiota using terminal restriction fragment length polymorphisms (T-RFLP), denaturing gradient gel electrophoresis (PCR-DGGE) and quantitative polymerase chain reaction (Q-PCR). DGGE bands potentially linked with SDG conversion to SECO, ED and EL were sequenced. Subsequently, pure culture studies using genera indicated from the sequences obtained by DGGE as being involved in the process, coupled with high pressure liquid chromatography (HPLC) for detection of antioxidants originating from SDG conversion were completed to ascertain their potential role in antioxidant production in the rumen. Thus, ultimately this study aimed to further our understanding of antioxidant production from SDG by identifying the ruminal bacteria that potentially play a role in this process.

Materials and Methods

Animals and treatments

All experimental procedures were approved by the local Animal Care Committee of the Dairy and Swine Research and Development Centre from Agriculture and Agri-Food Canada, Sherbrooke, Canada. Cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care [23]. Eight lactating multiparous Holstein cows fitted with ruminal cannulas averaging 686±33 kg of body weight and 112±21 days in milk were assigned to four treatments in a double 4×4 Latin Square design with four 21-d periods (14-d adaptation and 7-d sampling). The cows were kept in individual stalls and had free access to water. Diets were offered in equal amounts twice daily at 0830 and 1530 h for ad libitum intake [10% refusals as served] and milked twice daily in their stalls at 0800 and 1900 h. Cows were fed a total mixed ration (TMR; Table 1) with no FM (control, CON), or diets containing (DM basis) 5% FM, 10% FM (10FM) and 15% FM (15FM). The four total mixed diets were equal in protein and energy of lactation and were formulated to meet nutrient requirements for cows that average 657 kg of body weight and produce 37.7 kg/d of milk with 3.8% of fat, according to NRC [24].

Sampling and preparation

On Day 21 of each period, ruminal contents were collected 0, 2, 4 and 6 h after the morning meal from different locations within the rumen (the cranial dorsal, cranial anterior ventral, medium ventral, caudal dorsal and caudal ventral locations) to obtain a representative sample. The ruminal contents were strained through four layers of cheesecloth. One portion was kept at −20°C and freeze-dried for EL analysis. Another portion of 1L of strained ruminal fluid was taken 2 h post-feeding for microbial isolation as described by Lee et al. [25]. The resultant microbial pellets were freeze-dried, ground with a mortar and frozen at −80°C for molecular analysis.

Enterolactone analysis

EL analysis of rumen samples obtained from the animal trial was performed using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). The detailed procedures of extraction and analysis were described by Gagnon et al. [18]. Ruminal samples for the three post-feeding times (2, 4 and 6 h) were pooled within cow and period as previously carried out by Gagnon et al. [18] to obtain only one composite sample for EL analysis.

DNA extraction

DNA was extracted from approximately 10 mg of freeze-dried rumen samples using the FastDNA Spin Kit for Soil (QBiogene, Cambridge, UK) following the manufacturer’s guidelines, although 3×30S bead beating with 1 min intervals on ice was employed. The quality and quantity of DNA were determined using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA) (260 and 280 nm).

PCR-T-RFLP analysis of the total bacterial population

PCR T-RFLP was conducted as described by Huws et al. [26,27] in triplicate with subsequent pooling and use of the restriction enzymes Hae III or Msp I (Promega, Madison, USA). Restriction digested were run on an ABI3130xl DNA sequencer (Applied Biosystems, CA, USA) and T-RFs checked and exported using Genemapper software (Applied Biosystems, CA, USA). Peaks <0.5% of the cumulative peak height were removed [28]. Data were imported into Bio-Rad fingerprinting (Bio-Rad, Hertfordshire, UK) and clustering analysis was undertaken using separation criteria based on a 0.5 bp size difference in peaks and the Pearson’s coefficient.
In vitro incubations and HPLC analysis

In order to check whether the bacteria indicated as having a role in lignan metabolism in situ were actually capable of these biochemical conversions, we set up pure culture in vitro studies. Frozen-stocks of Butyrylribio fibosolvens JW 11, Butyrylribio proteotecticus B316, Eubacterium ruminantium 2398, Fibrobacter succinogenes S85, Peptostreptococcus anaerobius 27337, Prevotella albensis M 384, Prevotella bryantii B14, Prevotella ruminicola ATCC 19189, Ruminococcus albus SY3, and Ruminococcus flavefaciens 007 were obtained from the bacterial culture collection of the Institute of Biological, Environmental and Rural Sciences, Aberystwyth University. Pure cultures were grown anaerobically at 39°C for 24 h in Hobson’s Medium M2 [34], where 1 ml of each pure culture was added into 9 ml of broth within hungate tubes.

Firstly, stock solutions were prepared: SDG (1.45 nM) in water; SECO (2.75 nM), ED (3.30 nM) and EL (3.35 nM) in methanol. SDG, SECO, ED, EL and β-glucuronidase were obtained from Sigma-Aldrich Company Ltd (Dorset, United Kingdom). To test for the conversion of SDG into SECO, 25 µl of SDG stock solution, 5 µl of β-glucuronidase and 870 µl of Hobson’s Medium before addition of pure culture (100 µl) were placed into sterile hungate tubes and purged with CO₂ before closing. Experiments were conducted in duplicate and negative controls with bacteria but without SDG and positive controls with SDG without any bacteria were also analyzed. To test the conversion of ED into EL, 15 µl of ED stock solution were added to 885 µl of Hobson’s Medium. All other procedures were as described for conversion of SDG into SECO. As a further positive control samples containing 100 µl of sieved rumen fluid instead of any pure bacterial culture were added with SDG, SECO or ED respectively, again in duplicate. The rumen fluid for the in vitro work was obtained from 5 cannulated cows housed at IBERs Aberystwyth University and subsequently pooled for the experiment. All animal experiments are conducted under the authorities of the UK Animal (Scientific Procedures) Act (1986). The tubes were immediately incubated for 24 h at 39°C. Subsequently, incubations were centrifuged (1 min, 13,000 g). The supernatant was partially purified on Sep-Pak C₁₈.
Statistical Analysis

Band and peak numbers from TRFLP, Q-PCR and EL concentration were analyzed using the MIXED procedure of SAS (2000, SAS Institute, Cary, NC, USA). Treatment 5% FM was excluded for molecular bacterial analysis (PCR-DGGE, PCR-T-RFLP and Q-PCR) as the main objective was to compare the microbiota present under the control treatment versus high inclusions of FM in the diet (10% and 15%). Therefore, for the statistical analysis of DGGE band numbers, the number of T-RFLP peaks, and Q-PCR, the statistical model was a double incomplete 4×4 Latin square design while a double complete 4×4 Latin square was considered for EL concentration with the general model:

\[ Y_{ijklm} = \mu + T_i + P_j + Q_k + A_i/Q_k + e_{ijkl} \]

Where \( Y_{ijklm} \) is the response variable, \( \mu \) = overall mean, \( T_i \) = global effect of treatment (1 = CON, 5FM, 10FM and 15FM), \( P_j \) = the fixed effect of period (j = 1 to 4), \( Q_k \) = fixed effect of square (k = 1, 2), \( A_i/Q_k \) = random effect of cow within square, and \( e_{ijkl} \) = residual error. Enterolactone data were transformed (\( \log \)) as performed by Nesbitt et al. [35] due to the lack of variance homogeneity and variation in its concentration. However, the results in the Fig. 2 were expressed on the original scale of measurements. When a tendency was observed for an interaction (\( P \leq 0.10 \)) between treatment and time, the effect of treatment was examined within each time group, and then the treatment effects were compared at the relevant time. Normality and homogeneity were analyzed with the procedure UNIVARIATE of SAS (2000, SAS Institute, Cary, NC, USA). Statistical differences were declared at \( P < 0.10 \).

Results

Diet composition

Flax meal was fed at 4.79, 9.53 and 14.06% of dry matter, respectively, for treatments with 5, 10, and 15% of FM (Table 1). Concentrations of crude protein, acid-detergent fibre, neutral-detergent fibre and ether extract were similar among diets.

Ruminal concentration of EL

Flax meal supplementation increased concentration of EL linearly (\( P < 0.0001 \)) (Fig. 2) before feeding and in the pool of post-feeding times. However, there was an interaction (\( P = 0.1055 \)) between diet and sampling time for EL concentration in ruminal fluid as a result of a greater increase between before and after feeding for cows fed 15% FM compared to those fed the other diets.

Bacterial 16S rRNA quantity and diversity

Total bacterial 16S rRNA concentrations averaged 6.46, 7.65, and 7.27 ng g\(^{-1}\) 16S rRNA (S.E. = 0.41) for treatments CON, 10FM, and 15FM, respectively, and they were similar (\( P = 0.1505 \)) among treatments.

T-RFLP-derived unweighted pair group method with arithmetic mean (UPGMA) dendrograms did not show any global clustering dependent on diet for neither Hae III nor MSP I (Fig. 3A and 3B) potentially due to the fact that basal animal variation was high. A higher number of peaks for restriction enzyme MSP I was observed for cows supplemented with FM, but no treatment effect was observed with respect to the number of peaks obtained from Hae III (Table 2).

DGGE was employed as often data obtained using both techniques differ and also it is easier to subsequently sequence DGGE bands. When all cows were considered, there was no clear clustering by treatment likely due to the individual variation among animals as also noted in the T-RFLP analysis (Fig. 4A). However, when each cow was observed individually, four animals clustered closely based on diets (approx. 68% similarity seen between bacterial diversity present on the CON and 15FM diets; Fig. 4B), suggesting a treatment effect on the bacterial population (Fig. 5). These animals had an initial diversity which was far more similar compared with the other cows on trial. Thus, in order to investigate and identify which bacteria may be involved in SDG conversion and consequently EL production, the DNA extracted from bands present when the FM diet (15%) was fed compared to the CON diet were cut and sequenced on an individual animal from bands present when the FM diet (15%) was fed compared to the CON diet were cut and sequenced on an individual animal from bands present when the FM diet (15%) was fed compared to the CON diet.

In vitro experiment

Based on the \( in \ situa \) data, culturable bacteria belonging to the same genera found with DGGE were chosen to further probe the...
ability of these bacteria to metabolize SDG. In addition, species available in IBERS collection of the genus Butyrivibrio sp., Eubacterium sp. and Ruminococcus sp. were also selected for our in vitro experiments as they are known to play a role in the metabolism of lignans in humans [10,13,14] and goats [17]. After anaerobic incubation with SDG, we found using HPLC that all of the 11 bacteria investigated were able to hydrolyze the sugar portions of SDG and release SECO, namely the deglycosylation reaction [13].

Figure 3. PCR-T-RFLP-derived unweighted pair group method with arithmetic mean (UPGMA) dendograms showing the effect of flax meal inclusion on the rumen microbiota. The T-RFLP was based on Hae III (A) and MSP I (B) restriction enzymes. Cows were fed a control diet (CON) or a diet with 10% (10% FM) and 15% flax meal (15% FM). Scale relates to percent similarity and data are presented per period (Per). doi:10.1371/journal.pone.0087949.g003
mol), and expressed as the percentage of remaining SDG after 24 h of incubation and as SECO production after 24 h of incubation in relation to the initial concentration of SDG of 1.14 mM (blank without incubation). Each bacterium presented different efficiencies in ability to convert SDG to SECO, with *Prevotella* spp. being the most efficient and *B. fibrosolvens*, *P. anaerobius* and *F. succinogenes* having similar capacities to convert SDG into SECO (Table 4).

**Table 2.** Mean peak/band number following HaeIII- and MSP1-based 16S rRNA T-RFLP and V6–V8 PCR-DGGE of rumen bacteria within rumen samples obtained from Holstein cows fed a control diet with no flax meal (CON), 10% (10FM) and 15% (15FM) of flax meal (FM) in the dry matter.

| Treatments  | S.E.M. | P-value |
|-------------|--------|---------|
|             | CON    | 10FM    | 15FM    |
| Hae III     | 101.75 | 94.12   | 97.12   | 4.26    | 0.5222  |
| MSP I       | 75.62  | 99.62   | 82.25   | 3.79    | 0.0027  |
| PCR-DGGE    | 46.87  | 42.25   | 40.75   | 2.4     | 0.2149  |

Figure 4. PCR-DGGE-derived unweighted pair group method with arithmetic mean (UPGMA) dendograms showing the effect of flax meal inclusion on the rumen microbiota. The 16S rRNA PCR-DGGE is shown for all cows (A) and within four cows (B) which clustered based on the diet. Cows were fed a control diet (CON) or a diet with 10% (10% FM) and 15% flax meal (15% FM). Scale relates to percent similarity and data are presented per period (Per). doi:10.1371/journal.pone.0087949.g004
Neither ED nor EL was observed on the HPLC chromatograms after 24 h of incubation when the pure cultures were added with SDG as substrate. In addition, when ED was added as substrate, none of the 11 studied bacteria were able to convert ED into EL, and only ED peaks were identified on HPLC chromatograms after 24 h incubation (data not shown). In the positive control samples (SDG or ED incubated without any bacteria) SDG and ED concentration did not alter after incubation. The negative control samples (bacteria without any substrate) did not show any presence of lignans after incubation. However, when ruminal fluid was used as inoculum, positive results were observed, as for conversion of SDG into SECO and ED (Table 4); and for conversion of SECO into ED and conversion of ED into EL (data not shown).

Discussion

The study was designed to identify the ruminal bacteria responsible for the conversion of plant lignans into mammalian lignans. This study indicates that many bacteria, in particular the Prevotella spp. genus, may be important for converting plant into mammalian lignans in the rumen. Previous results have demonstrated that the main site of mammalian lignan formation in ruminant animals was the rumen [18]. Indeed, increased levels of the mammalian lignan EL in ruminal fluid of cows supplemented with flax hulls [7,36] and sheep infused with purified SDG in the rumen [17] have been reported earlier. Feed ingredients such as soy and corn contain lignans [37,38] also lead to EL production [39], which may explain why EL was present in the rumen of cows fed CON. However, FM was clearly the main source of lignans (i.e., SDG) in the diet as it is known to be one of the richest sources of plant lignans [39].

T-RFLP indicated no overall change in the bacterial communities with FM supplementation for both Hae III and MSP1 endonucleases, even when animals were compared separately due to the basal variation in the rumen microbiota highlighted by T-RFLP. Nonetheless, overall changes were observed in the ruminal microbiota upon FM feeding when the PCR-DGGE technique was applied when cows were analyzed separately. Indeed, four animals clustered closely based on diets and distinct bands appeared when FM was supplemented. Discrepancies between T-RFLP and DGGE data have previously been noted, which may be because a different amplicon is used for both techniques [40–42]. For the purpose of investigating and identifying which bacteria may be involved in SDG metabolism, the DNA extracted from bands of interest present when the 15% FM diet was fed compared to the CON diet were cut and sequenced on an individual animal basis. The sequencing data obtained indicated which rumen bacteria could potentially be responsible for metabolizing the flax lignans. The subsequent in vitro incubations and HPLC analysis confirmed that the 11 ruminal species of bacteria selected based on our data and those of monogastric trials were able to convert SDG into SECO, which is a deglycosylation reaction catalyzed by the enzyme β-glucuronidase [43]. In the present experiment, although β-glucuronidase had been added to SDG to allow the deglycosylation reaction to occur during the 24 h incubation, each strain possessed different capacities to metabolize SDG into SECO. Bacteria from genera Prevotella presented a higher efficiency of conversion of SDG into SECO, followed by B. fibrisolvens and P. anaerobius. Conversely, R. albus, E. ruminantium and R. flavefaciens were less efficient in SDG conversion compared with Prevotella spp. B. proteoclasticus also showed low SECO production and the lowest recovery of both plant lignans (SDG and SECO), suggesting that intermediate compounds other than ED and EL were obtained during the metabolism of plant SDG. Indeed, recovery of the substrate and the metabolites SECO, ED and EL investigated in this experiment did not reach 100%. Wang et al. [11] identified seven metabolites of SDG when incubating SDG with human faeces. For example, matairesinol is endoglucanases, cellobiosidase, cellodextrinase, xylanases, and important role in lignan conversion within the rumen.

The genus Bacteroides, which belongs to the order Bacteroidales, was identified by Clavel et al. [8] as one of the human intestinal bacteria responsible for the catalytic conversion of SDG to SECO via deglycosylation (β-glycosidases). The family Prevotellaceae and the genus Prevotella also belong to the order Bacteroidales. As the genus Prevotella within the family Prevotellaceae represented more than 45% of the identified bands in the current study and were the main SECO producers in vitro, this may suggest that they play an important role in lignan conversion within the rumen. F. succinogenes is a fibrolytic bacterium and its enzymes possess endoglucanases, cellobiosidase, cellobioextrinase, xylanases, and β-glucoisidase activities [46]. In the present experiment, F. succinogenes S85 was able to convert SDG into SECO. This agrees with the activity of β-glucoisidase that has been demonstrated by F. succinogenes S85 [46]. Indeed, β-glucoisidase is the enzyme required.
for the conversion of SDG to SECO [14]. In a recent study, Zhou et al. [17] found that Ruminococcus gnavus was potentially responsible for the conversion of plant lignans into EL in goats. In the current study, it was shown that R. albus and R. flavefaciens also were involved in the conversion of SDG to SECO.

Table 3. Taxonomic identification of DGGE bands potentially associated with enterolactone production in ruminal fluid.

| Band position (clone no.) | Nearest match (accession number; Maximum% sequence similarity) | Ribosomal Database Project Classification |
|--------------------------|---------------------------------------------------------------|------------------------------------------|
| Cow 1 (band position A clone 1) | Uncultured bacterium isolate 16S ribosomal RNA gene (EU624093.1; 99%) | unclassified_Succinivibrionaceae |
| Cow 3 (band position A clone 1) | Uncultured rumen bacterium clone CF23 16S ribosomal RNA gene (EU871348.1; 94%) | unclassified_Lachnospiraceae |
| Cow 3 (band position A clone 2) | Uncultured bacterium clone p-1030-a5 16S ribosomal RNA gene (AF371866; 96%) | unclassified_Alphaproteobacteria |
| Cow 4 (band position A clone 1) | Uncultured rumen bacterium clone YRC13 16S ribosomal RNA gene (EU259389.1; 98%) | unclassified_Bacteroidales |
| Cow 4 (band position B clone 1) | Uncultured rumen bacterium clone BF399 16S ribosomal RNA gene (EU850583.1; 96%) | genus Anaerovorax |
| Cow 4 (band position B clone 2) | Uncultured rumen bacterium clone PS_D21 16S rRNA (AB034106.1; 99%) | genus Succinivibrio |
| Cow 4 (band position B clone 3) | Uncultured rumen bacterium clone YRC13 16S ribosomal RNA gene (EU259389.1; 98%) | genus Fibrobacter |
| Cow 4 (band position B clone 4) | Uncultured rumen bacterium clone P5_D21 16S ribosomal RNA gene (EU381799.1; 98%) | genus Prevotella |
| Cow 4 (band position C clone 1) | Uncultured rumen bacterium clone TWBR864 16S ribosomal RNA gene, partial sequence (FJ028779.1; 94%) | unclassified_Prevotellaceae |
| Cow 4 (band position C clone 2) | Uncultured rumen bacterium clone BE5 16S ribosomal RNA gene (AY244922.1; 96%) | genus Prevotella |
| Cow 4 (band position C clone 4) | Uncultured bacterium clone P5_D21 16S ribosomal RNA gene (EU381799.1; 98%) | genus Fibrobacter |
| Cow 5 (band position A clone 1) | Uncultured bacterium clone CTRS1H03 16S ribosomal RNA gene (GQ327793.1; 97%) | genus Prevotella |

Table 4. Efficiency of conversion of SDG into SECO by selected pure cultures of ruminal bacteria and conversion of SDG into SECO and ED using ruminal fluid as inoculum, assessed using in vitro cultures and HPLC1.

| % SDG remaining after 24 h incubation | % of SECO produced based on initial SDG amount | % of ED produced based on initial SDG amount |
|--------------------------------------|---------------------------------------------|---------------------------------------------|
| Prevotella bryantii                   | 2.1                                         | 81.7                                        | n.d.                                        |
| Prevotella albenssi                   | 2.1                                         | 60.4                                        | n.d.                                        |
| Prevotella ruminicola                | 4.8                                         | 56.5                                        | n.d.                                        |
| Prevotella brevis                     | 44.9                                        | 49.4                                        | n.d.                                        |
| Peptostreptococcus anaerobius        | 20.5                                        | 49.2                                        | n.d.                                        |
| Butyrivibrio fibrosolvens            | 26.8                                        | 50.8                                        | n.d.                                        |
| Fibrobacter succinogens              | 33.3                                        | 39.1                                        | n.d.                                        |
| Ruminococcus albus                   | 60.6                                        | 14.9                                        | n.d.                                        |
| Eubacterium ruminantium              | 79.1                                        | 11.8                                        | n.d.                                        |
| Butyrivibrio proteoelacticus         | 39.9                                        | 6.8                                         | n.d.                                        |
| Ruminococcus flavefaciens            | 76.9                                        | 3.3                                         | n.d.                                        |
| Ruminal fluid                        | 2.1                                         | 46.6                                        | 8.0                                         |

1The HPLC results were converted into molarities (SDG M = 686.7 g/mol; SECO M = 362.4 g/mol; ED M = 302.36 g/mol), and expressed as the percentage, in relation to the initial concentration of SDG of 1.14 mM; n.d.: non-detected.

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adapted to convert SDG. This agrees with the results of Antignac et al. [47] who observed a large variation in milk EL concentration as a response to animal feeding. In addition, Höjer et al. [48] also reported large variations in equal concentration between individual cows, indicating the possibility of selecting high or low producers of EL. Similar results have been observed by Petit et al. [unpublished data] for high and low producers of EL. The lack of in vitro production of EL from SDG also could indicate that bacteria metabolizing ED into EL did not grow well under the present experimental conditions. Indeed, previous results have shown no conversion [11] or conversion [49,50] of ED into EL after in vitro incubation of SDG with a rat faecal suspension. In addition, Borriello et al. [51] reported that there was no conversion of ED into EL when no viable bacteria were presented in diluted human faeces used as inoculum. Thus, the differences in outcome of ED conversion to EL in these published experiments are likely linked to presence of lower colonic bacteria [52]. Conversely, previous results from Córtes et al. [7] have shown that EL formation occurs when ruminal fluid from cows fed flax products (hulls and seeds) was used as inoculum. Discrepancies between studies could also be due to other factors such as differences in the physical form of the substrate. Indeed, purified SDG was added in the present trial and not flax meal.

In summary, inclusion of FM increased concentration of the mammalian lignan EL in the rumen and altered the ruminal microbiota as demonstrated using DGGE. Sequencing of key bands present upon inclusion of 15% FM in the diet and absent when no FM was fed showed that diverse ruminal bacterial taxa may play a role in the metabolism of flax lignans. Subsequent in vitro studies supported the in situ data and showed that Prevotella spp. in particular may contribute to the conversion of SDG into SECO in the rumen. However, further studies are required to identify ruminal bacteria responsible for the formation of ED and EL. Identification and characterization of the enzymes involved in the conversion of plant into mammalian lignans, which are linked to better human health, is also paramount for developing animal products enriched in enterolactone.

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Author Contributions

Conceived and designed the experiments: ALBS SAH GTDS NDS BDH ALW EJK HVP. Performed the experiments: ALBS SAH GTDS NDS BDH ALW EJK HVP. Analyzed the data: ALBS SAH GTDS BDH ALW EJK HVP. Contributed reagents/materials/analysis tools: ALBS SAH GTDS BDH ALW EJK HVP. Wrote the paper: ALBS SAH GTDS NDS BDH ALW EJK HVP.

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