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ORIGINAL ARTICLE

Fatty acid oxidation products ('green odour') released from perennial ryegrass following biotic and abiotic stress, potentially have antimicrobial properties against the rumen microbiota resulting in decreased biohydrogenation

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bacteria, biohydrogenation, fatty acid, green odour, hydroperoxides, jasmonic acid, rumen, salicylic acid.

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Abstract

Aims: In this experiment, we investigated the effect of 'green odour' products typical of those released from fresh forage postabiotic and biotic stresses on the rumen microbiota and lipid metabolism.

Methods and Results: Hydroperoxyoctadecatrienoic acid (HP), a combination of salicylic and jasmonic acid (T), and a combination of both (HPT) were incubated *in vitro* in the presence of freeze-dried ground silage and rumen fluid, under rumen-like conditions. 16S rRNA (16S cDNA) HaeIII-based terminal restriction fragment length polymorphism-based (T-RFLP) dendrograms, canonical analysis of principal coordinates graphs, peak number and Shanon-Weiner diversity indices show that HP, T and HPT likely had antimicrobial effects on the microbiota compared to control incubations. Following 6 h of *in vitro* incubation, 15·3% of 18:3*n-3* and 4·4% of 18:2*n-6* was biohydrogenated in control incubations, compared with 1·3, 9·4 and 8·3% of 18:3*n-3* for HP, T and HPT treatments, respectively, with negligible 18:2*n-6* biohydrogenation seen. T-RFLP peaks lost due to application of HP, T and HPT likely belonged to as yet uncultured bacteria within numerous genera.

Conclusions: Hydroperoxyoctadecatrienoic acid, T and HPT released due to plant stress potentially have an antimicrobial effect on the rumen microbiota, which may explain the decreased biohydrogenation *in vitro*.

Significance and Impact of the Study: These data suggest that these volatile chemicals may be responsible for the higher summer *n-3* content of bovine milk.

Introduction

With a population set to double in size in the next 40 years, the need to ensure affordable, accessible, nutritionally and microbiologically safe ruminant products for the future has never been as important (Foresight 2011). The rumen microbiota are central to these challenges as their utilization of proteins and lipids defines ruminant use efficiency and product quality (Harfoot and Hazlewood 1997). In the light of these challenges, there is a need to increase our understanding of the rumen ecosystem in order to develop novel methodologies of ensuring food security from an agricultural and consumer perspective.

Forages ingested by ruminants are commonly rich in human health beneficial polyunsaturated fatty acids (PUFAs), particularly 18:3*n-3*, yet meat and milk are high in nutritionally detrimental saturated fatty acids (SFAs) with the PUFA/SFA ratio being <0·4 (Scollan *et al.* 2006; Kim *et al.* 2008; Huws *et al.* 2010; Lourenço *et al.* 2010). This is due to the fact that the rumen microbiota biohydrogenate dietary PUFA to SFA, producing conjugated diene and triene intermediates (Wąsowska *et al.* 2006; Jenkins *et al.* 2008; Kim *et al.* 2008; Huws *et al.* 2010;

Lourenço *et al.* 2010), which have also been shown to have important health beneficial properties (Lock and Bauman 2004; Tsuzuki *et al.* 2004). Current evidence suggests that many as yet uncultured rumen bacteria belonging to the families: *Prevotella*, *Lachnospiraceae* incertae sedis and unclassified: *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* have biohydrogenating capacity (Boeckaert *et al.* 2008; Kim *et al.* 2008; Belenguer *et al.* 2010; Huws *et al.* 2011). In recent years, much emphasis has been given to developing novel strategies of controlling biohydrogenation to enhance the health benefits of ruminant products for the consumer. Nevertheless, developing targeted approaches towards the as yet unculturable taxa is challenging.

It is well known that grass-fed cows as opposed to those fed conserved forages produce milk with less saturated fatty acids and more conjugated dienes (Dhiman *et al.* 1999; Offer 2002; Dewhurst *et al.* 2006; Lee *et al.* 2007). Fatty acid oxidation products are produced by the plant during biotic and abiotic stress and are commonly called 'green odour'. When they are released during mastication, such compounds may provide a potential explanation for decreased SFA levels in the milk of animals grazing fresh pasture (Dewhurst *et al.* 2006; Lee *et al.* 2007). Indeed, we have previously shown that the fatty acid oxidation product 1,2-dimethyl hydroperoxide (1,2-DMEH), change ruminal bacterial diversity whilst increasing biohydrogenation diene and triene intermediate formation as a potential reason for higher levels of conjugated linoleic acid in pasture-based milk (Lee *et al.* 2007). However, although 1,2-dimethyl hydroperoxide (1,2-DMEH) is a typical hydroperoxide, it is not specific to compounds that are released during the damage of living plant tissue – green odour (Lee *et al.* 2007). In this experiment, we investigated the effect of a hydroperoxide (initial oxidation products of 18:3*n-3*) (Hydroperoxyoctadecatrienoic acid, HP; 200 μmol l^{-1}), terminal green odour compounds (a combination of salicylic and jasmonic acid, which are commonly released due to plant damage, T; 200 μmol l^{-1}), and a combination of both (50 : 50; HP : T; HPT; 200 μmol l^{-1}) at concentrations based on release rates of cut grass (Hatanaka 1993) on the metabolically active rumen microbiota (RNA used as a marker) and C18 fatty acid metabolism *in vitro*.

Materials and methods

Experimental design

Freeze-dried and ground grass (*Lolium perenne*) silage (1 g DM) was added to extraction tubes (20 ml total volume) together with anaerobic incubation buffer (6 ml prewarmed to 39°C; Van Soest 1967) and rumen fluid inoculum (6 ml, strained through two layers of muslin and held under CO_2 at 39°C). Rumen inoculum was produced by combining equal amounts of strained rumen fluid and extracted solid digesta [12% in Goering and Van Soest (1970) buffer] obtained from three fistulated dairy cows fed on grass silage (Lee *et al.* 2007). Triplicate incubations were set up in the absence of treatment (control) and presence of a hydroperoxide (Hydroperoxyoctadecatrienoic acid, HP, 200 μmol l^{-1}), terminal green odour compounds (an equal combination of salicylic (Sigma-Aldrich, Dorset, UK) and jasmonic acid (Sigma-Aldrich), which are commonly released due to plant damage, T, 200 μmol l^{-1}) and a combination of both (50 : 50; HP : T; HPT, 200 μmol l^{-1}). The hydroperoxide was prepared from linolenic acid as described by Drouet *et al.* (1994) and Gardner and Grove (2001). Essentially, linolenic acid (100 mg) was dissolved in octane, which was subsequently added to a LOX-1 enzyme (Sigma-Aldrich) in borate buffer (pH 9·6) at a ratio of 1 : 8, octane/buffer. The subsequent reaction was catalysed by oxygen, which was added through a diffuser with vigorous stirring for 30 min. Reaction was stopped by adding ethanol until solution had a final amount of 20% ethanol. Solution pH was lowered to 4·0 with 1 N HCl, and chloroform was subsequently added in similar volume to the HP solution and mixed vigorously for 1 min. Solution was centrifuged (1000 ***g*** for 5 min) and lower aqueous phase was removed and dried in a rotary evaporator. When dry, HP was immediately resuspended in ethanol and stored at −20°C until experiment was conducted. The HP derived from this procedure was 13-Hydroperoxyoctadecatrienoic acid as the LOX enzyme used was specific for formation of this particular HP. Post-setting up the treatments, bottles were incubated in a horizontally rotating rack at 100 rpm and 39°C and bottle contents were harvested in triplicate at 0, 2, 4 and 8 h. Essentially, bottle contents were blended at the designated time points, and aliquots were taken for RNA-based microbial analysis, whilst the rest was frozen in liquid nitrogen before placing into −20°C and freeze-drying prior to lipid extraction and analysis. Remaining dry matter in the bottles post freeze-drying was also recorded.

RNA extraction

RNA was extracted from blended contents of the extraction bottles (200 μl) using the BIO101 FastRNA Pro Soil-Direct Kit (QBiogene, Cambridge, UK) following the manufacturer's guidelines. DNA was removed from extracted RNA using RNase-free DNase (Promega, Southampton, UK) treatment according to manufacturer's guidelines. The quality and quantity of RNA were determined using a spectrophotometer (260 and 280 nm) and

using the Experion System (Bio-Rad UK Ltd, Hemel Hempstead, UK).

Preparation of cDNA

RNA (*ca*.100 ng) was reverse-transcribed using the reverse primer R1401 (Huws *et al.* 2007, 2011) and Superscript III reverse transcriptase (Invitrogen Ltd., Paisley, UK) in 20 μl reactions, following the manufacturer's guidelines and as described by Edwards *et al.* (2007) and Huws *et al.* (2011). Control reactions were performed with no reverse transcriptase to confirm that the RNA preparations were free of contaminating DNA.

Terminal restriction fragment length polymorphism (T-RFLP)

Amplification of 16S rRNA was accomplished using primers 27f (FAM labelled on 5′ end) 5′- AGA GTT TGA TCC TGG CTC AG-3′ and 1389r 5′-ACG GGC GGT GTG TAC AAG-3′ as described by Huws *et al.* (2011), although in this instance, 3 × 25 μl PCRs were performed. PCR amplicons were pooled for each sample before purification using the Qiagen PCR purification kit (Qiagen, West Sussex, UK). Purified PCR products were digested using HaeIII, MSPI or AluI for 5 h at 37°C. Terminal restriction fragments (T-RFs) were subsequently separated based on size using the Applied Biosystems ABI3130xl sequencer (Life Technologies Ltd., Paisley, UK) using GSLiz 600 size standard (Applied Biosystems). TRFs were viewed using GeneMapper (Applied Biosystems), and data were exported into Microsoft Excel and formatted appropriately for import into the Bio-Rad fingerprinting software (Bio-Rad, Hertfordshire, UK). Based on background peak levels found within the nondigested PCR controls, peaks <50 fluorescence units were removed. Data were imported into Bio-Rad fingerprinting (Bio-Rad), and clustering analysis was undertaken using a differential band tolerance equating to 0·5 bp and the Pearson coefficient. The binary data generated were used to calculate band number and Shannon's diversity indices as described under statistical analysis. TRFs were aligned using T-align (Smith *et al.* 2005) and subsequently analysed using Primer6 and PERMANOVA+ (version 6; Primer-E, Ivybridge, UK) as described under statistical analysis. Putative identification of important TRFs was completed using the Ribosomal Database Project (RDP) with the aid of RDP staff (Cole *et al.* 2009).

Fatty acid analysis

Freeze-dried bottle contents were treated with 25 ml of isopropanol/chloroform (1 : 1 v/v) along with 1 ml of internal standard (2·5 mg C19:0 per ml chloroform) and extracted as described by Lee *et al.* (2007). The samples were then bimethylated using the procedure of Kramer and Zhou (2001) and analysed by gas chromatography on a CP-Select chemically bonded for FAME column (100 m × 0·25 mm I.D., Varian Inc., Yarnton, UK) with split injection (1 : 50). Peaks were identified from external standards (ME61, Laroden fine chemicals, Malmo, Sweden; S37, Supelco, Poole, Dorset, UK; CLAs, Matreya, Philadelphia, PA, USA) and quantified using the internal standard (C19:0).

Statistical analysis

All fatty acids for both experiments are reported as a percentage of total fatty acids in the incubations so that the results were not confounded by level of fatty acid within different incubation bottles. Fatty acid, dry matter degradation and peak number data were subjected to analyses of variance using Genstat (Payne *et al.* 2007). Primer6 and PERMANOVA+ (version 6; Primer-E) respectively were used to conduct canonical analysis of principal coordinates (CAP) (Anderson and Willis 2003) and permutation multivariate analysis of variance (PERMANOVA) (Anderson 2001) on the TRF data. Shannon's diversity indices and comparative *t*-tests were calculated from the binary data exported from Bio-Rad fingerprinting using FAMD 1.25 software (Schlüter and Harris 2006).

Results

Bacterial diversity

HP, T and HPT combinations caused changes in the bacterial populations present, which was apparent consistently in dendrograms generated from HaeIII T-RFLP profiling methodologies (Fig. 1). Nonetheless, this clustering based on treatment was not as apparent in dendrograms generated from MSPI-based T-RFLP (Fig. 2a–c). MSPI-based T-RFLP gave the least amount of peaks consistently irrespective of treatment and time (Table 1), thus, the MSPI T-RFLP-based dendrograms may not be representative of the bacterial diversity present. Alu1-based T-RFLP was also conducted, but peak number retrieved from samples were even lower than with MSPI PCR digestions and in some cases absent making this information unsuitable for further analysis (data not shown).

HaeIII-based T-RFLP dendrograms showed subclustering of control and HP added microbiota for all time points (approx. similarity 38%; Fig 1a). The ruminal microbiota in the presence of T showed differences only in control and test incubation in the presence of T compounds up to 4 h of incubation post-HaeIII T-RFLP

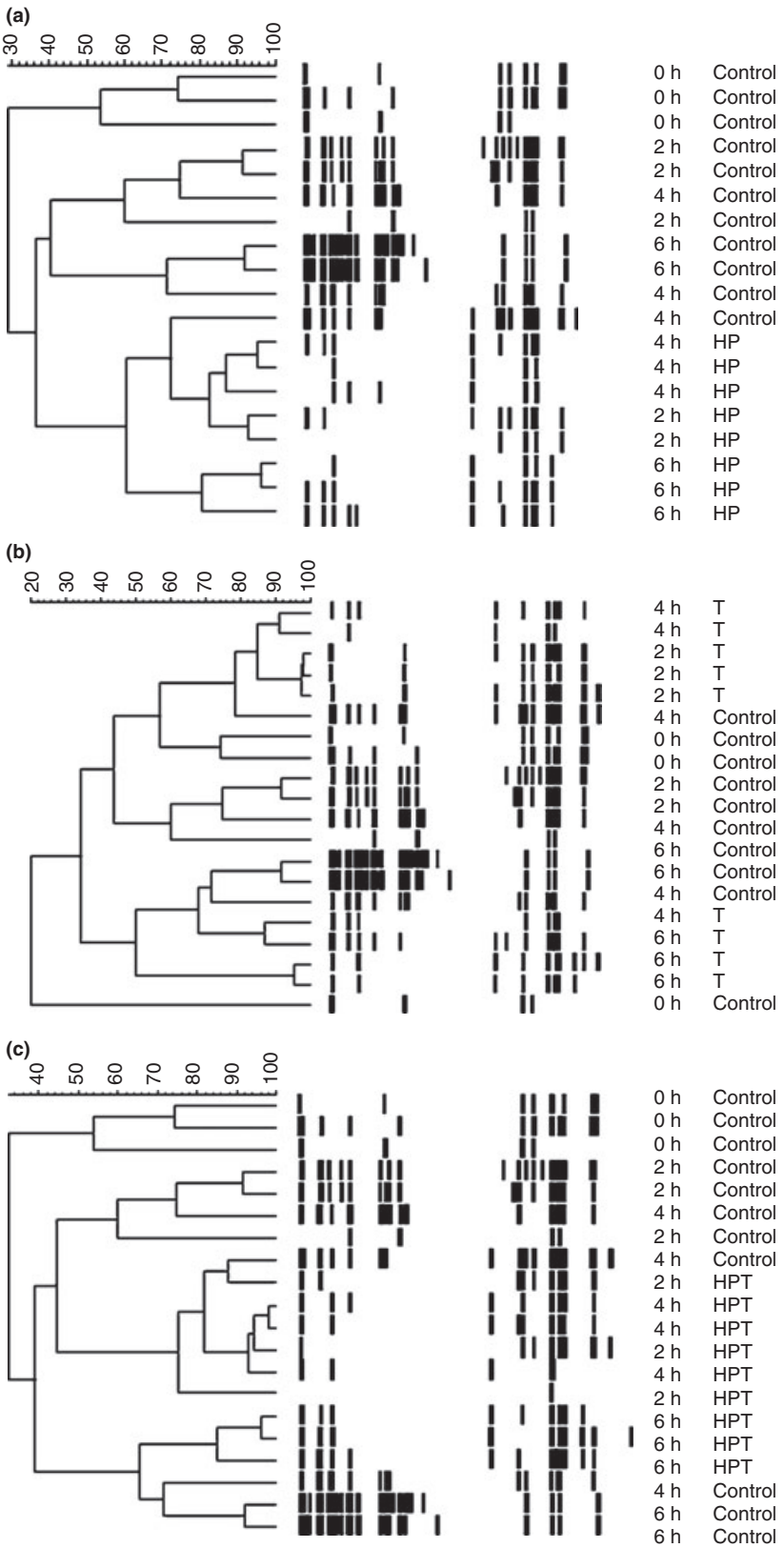

Figure 1 Unweighted pair group method with arithmetic mean dendrogram showing the effect of Hydroperoxyoctadecatrienoic acid (HP; 200 μmol l^{-1}) (a) salicylic acid and jasmonic acid (T; 200 μmol l^{-1}) (b) and Hydroperoxyoctadecatrienoic acid, salicylic acid and jasmonic acid (HPT; 200 μmol l^{-1}) (c) on the rumen microbiota using 16S rRNA (16S cDNA) and HaeIII-based T-RFLP compared to control incubations in the absence of any treatment. Scale relates to percent similarity.

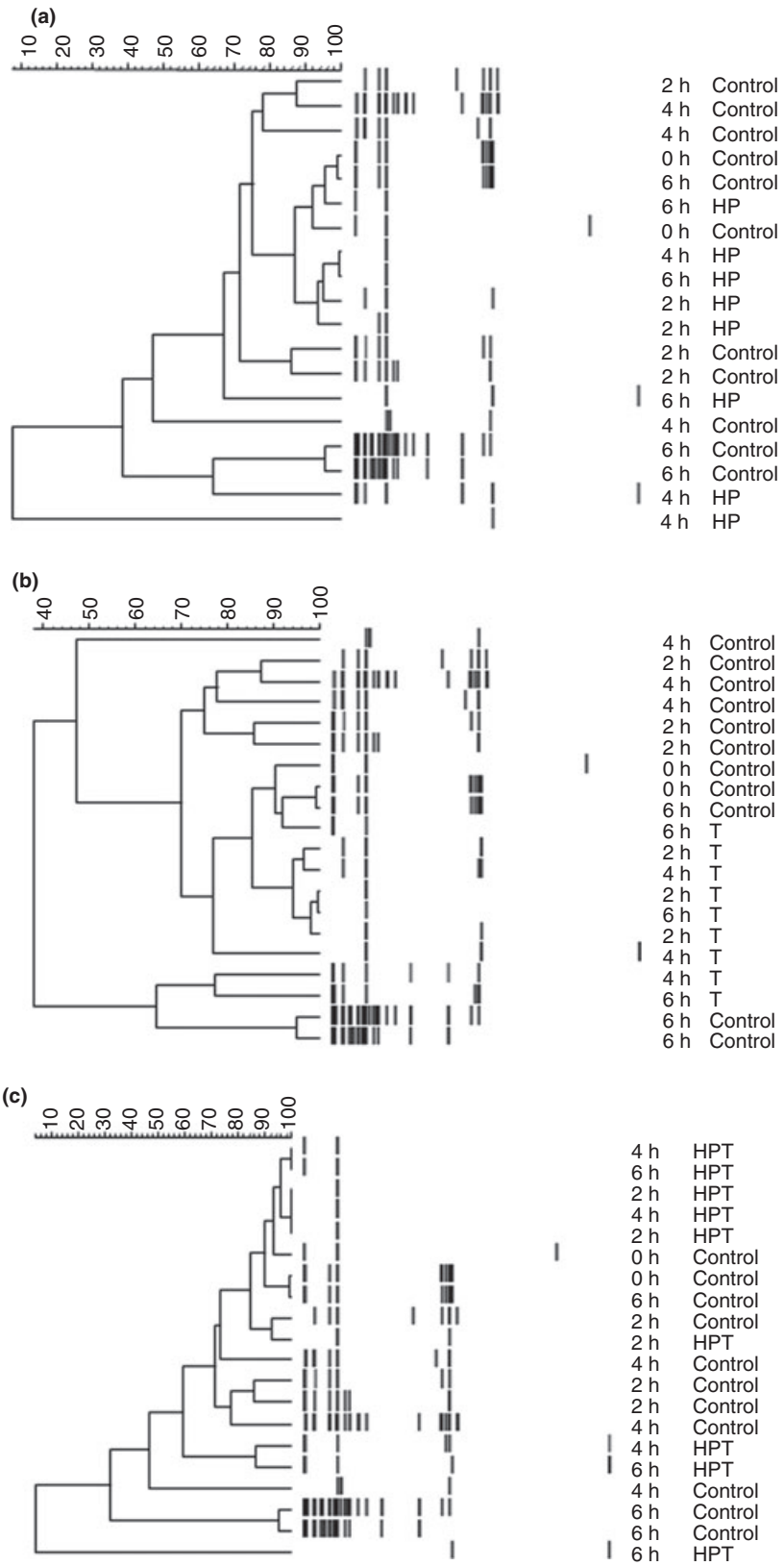

Figure 2 Unweighted pair group method with arithmetic mean dendrogram showing the effect of Hydroperoxyoctadecatrienoic acid (HP; 200 μmol l^{-1}) (a), salicylic acid and jasmonic acid (T; 200 μmol l^{-1}) (b) and Hydroperoxyoctadecatrienoic acid, salicylic acid and jasmonic acid (HPT; 200 μmol l^{-1}) (c) on the rumen microbiota using 16S rRNA (16S cDNA) and MSP1-based T-RFLP compared to control incubations in the absence of any treatment. Scale relates to percent similarity.

Table 1 Peak numbers and Shannon's diversity indices retrieved post-HaeIII-based T-RFLP from *in vitro* incubations in the absence of treatment (control), and presence of a hydroperoxide (Hydroperoxyoctadecatrienoic acid, HP, 200 μmol l^{-1}), terminal green odour compounds (a combination of salicylic and jasmonic acid, which are commonly released due to plant damage, T, 200 μmol l^{-1}) and a combination of both (50 : 50; HP;T; HPT, 200 μmol l^{-1})

	Time (h)		
	2	4	6
Peak number			
Control	20·67[b]	21·33[b]	33·00[b]
HP	6·50[a]	6·00[a]	8·67[a]
T	11·00[a]	7·33[a]	12·00[a]
HPT	11·00[a]	7·67[a]	12·67[a]
SEM	2·75	3·00	2·88
P	0·007	0·003	<0·001
Shannon-Weiner diversity index (*H'* variance)			
Control	4·20 (0·08)[a]	4·60 (0·05)[a]	4·18 (0·20)[a]
HP	2·32 (0·11)[b]	2·50 (0·35)[b]	2·84 (0·26)[b]
T	2·85 (0·27)[b]	3·01 (0·24)[b]	3·98 (0·10)[b]
HPT	2·92 (0·23)[b]	2·43 (0·34)[b]	2·84 (0·27)[b]

ANOVA was conducted for peak numbers at each time point to assess the effects of treatment at that time. Shannon's diversity indices and comparative *t*-tests were obtained using the FAMD software on Shannon's diversity indices (Schlüter and Harris 2006). Values with different superscripts on the within the same column differed significantly ($P < 0·05$).

(approx. similarity 46%), with 6 h samples clustering similarly (Fig 1b). Indeed similar clustering patterns were seen with addition of T compounds alone to those comparing control and addition of both HP and T compounds (Fig 1c). Band number and Shannon's diversity indices were significantly lower in the presence of HP, T and HPT, compared with control incubations in the absence of treatment, at all time points (Table 1).

Canonical analysis of principal coordinates (CAP) ordination on the basis of Pearson similarity matrices of HaeIII T-RFLP confirmed previous dendrograms (Fig 3a–c). PERMANOVA showed that HP did not affect the microbiota significantly ($P = 0·24$, *Pseudo-F* = 1·783), whilst T and HPT combinations did significantly alter the microbiota present within the incubations compared to control incubations ($P = 0·01$, *Pseudo-F* = 23·084 for T; $P = 0·003$, *Pseudo-F* = 10·204 for HPT).

Predictive phylogeny of peaks lost due to addition of HP, T and HPT

Aligned TRFs for 4 h data were analysed to detect peaks that were targeted by the 'green odour' volatiles. In essence, if 2/3 control incubations had a peak present, which was absent in all test incubations, it was

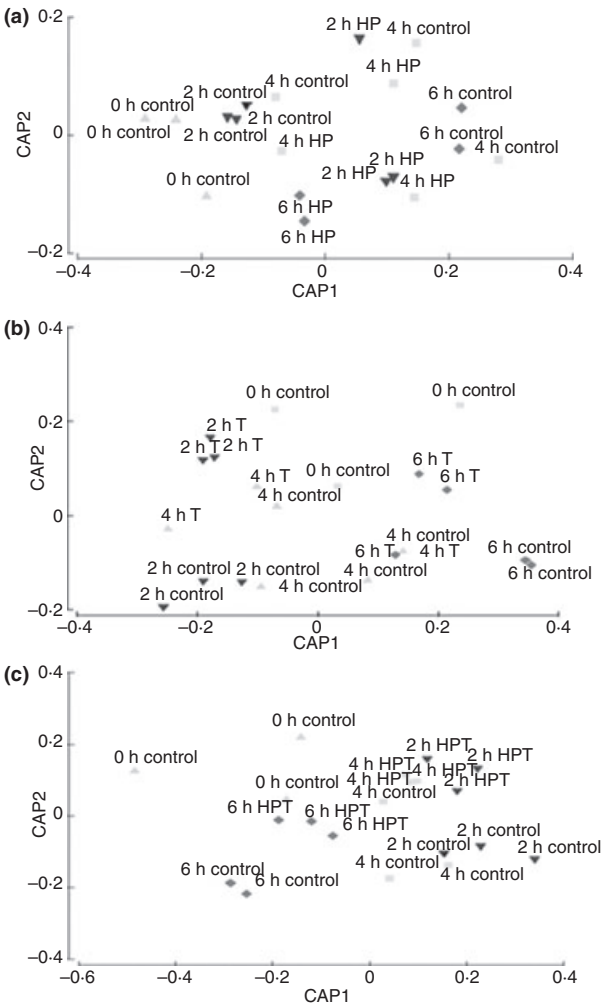

Figure 3 Canonical analysis of principal coordinates (CAP) ordination showing the effect of (a) Hydroperoxyoctadecatrienoic acid (HP; 200 μmol l^{-1}), (b) salicylic acid and jasmonic acid (T; 200 μmol l^{-1}) and (c) a mixture of hydroperoxides, salicylic acid and jasmonic acid (HPT; 200 μmol l^{-1}) on the on the rumen microbiota.

considered that bacteria pertaining to this TRF were inhibited by the 'green odour' volatile. Peaks lost due to addition of HP were 230, 231, 237, 266 and 298 bp in size and are likely attributed to as yet unculturable bacteria belonging to *Veillonellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Acetivibrio*, *Coprococcus*, *Clostridia*, *Anaeovibrio*, *Oscillibacter*, *Prevotella*, *Papillibacter* and *Leuconostoc* (Table 2). Peaks lost due to addition of T were 78, 230, 231 and 266 bp in size and are likely attributed to as yet unculturable bacteria belonging to *Veillonellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Acetivibrio*, *Coprococcus*, *Clostridia*, *Anaeovibrio*, *Prevotella*, *Papillibacter* and *Leuconostoc* (Table 2). Peaks lost due to addition of a combination of HPT were 230, 237 and 266 bp in size and are

Table 2 Sizes of terminal restriction fragments which are no longer present post exposure to a hydroperoxide (Hydroperoxyoctadecatrienoic acid, HP, 200 μmol l^{-1}), terminal 'green odour' compounds (a combination of salicylic and jasmonic acid, T, 200 μmol l^{-1}) and a combination of both (50 : 50; HP;T; HPT, 200 μmol l^{-1}) and their predictive taxonomy based on 4 h incubation data

Treatment	Terminal restriction fragment size (nearest bp)	Predictive terminal restriction fragment genus level identification*
HP	230	As yet uncultured unclassified *Veillonellaceae* and *Ruminococcaceae* and classified *Acetivibrio* and *Coprococcus*
	231	As yet uncultured unclassified *Clostridia* and *Veillonellaceae* and classified *Acetivibrio* and *Anaerovibrio*
	237	As yet uncultured unclassified *Veillonellaceae* and *Lachnospiraceae* and classified *Oscillibacter*
	266	As yet uncultured unclassified *Lachnospiraceae*, *Ruminococcaceae* and *Prevotellaceae*, and classified *Prevotella*, *Clostridium*, *Acetivibrio*, *Papillibacter* and *Leuconostoc*
	298	As yet unclassified uncultured *Lachnospiraceae* and *Ruminococcaceae* and classified *Ruminococcus* and *Acetivibrio*
T	78	As yet uncultured unclassified *Ruminococcaceae*
	230	As above
	231	As above
	266	As above
HPT	230	As above
	237	As above
	266	As above

*Generated using the Ribosomal Database Project (Cole *et al.* 2009). We are very grateful to RDP staff for completing the TRF taxonomic assignments for this paper.

likely attributed to as yet unculturable bacteria belonging to *Veillonellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Acetivibrio*, *Coprococcus*, *Clostridia*, *Anaeovibrio*, *Oscillibacter*, *Prevotella*, *Papillibacter* and *Leuconostoc* (Table 2). Thus, HP, T and HPT likely affect similar bacteria.

Biohydrogenation

Biohydrogenation of 18:3*n-3* and 18:2*n-6* through to 18:0 in the presence of HP was inhibited as early as 2 h postincubation (control had 7·7% less 18:3*n-3*, 3·8% less 18:2*n-6* and 28% more 18:0 present compared to incubations in the presence of HP; Table 3). Following 4 h of incubation, HP, T and HPT additions had all inhibited biohydrogenation of 18:3*n-3* and 18:2*n-6* through to 18:0 (controls had 16·4, 9·6 and 10·5% less 18:3*n-3* present than in the presence of HP, T and HPT, respectively; controls had 8·4, 3·5 and 4·2% less 18:2*n-6* present than in the presence of HP, T and HPT, respectively; controls had 53·0, 30·5 and 38·3% more 18:0 present than in the presence of HP, T and HPT, respectively; Table 3). At 6 h postincubation, only HP continued to have an inhibitory effect on the biohydrogenation of 18:3*n-3* and 18:2*n-6* (control had 7·7% less 18:3*n-3*, 7·5% less 18:2*n-6* and 28% more 18:0 present compared to incubations in the presence of HP; Table 3),whilst a combination of HPT reduced 18:3*n-3* biohydrogenation (control had 8·3% less 18:3*n-3* and 14% more 18:0 present compared to incubations in the presence of HP; Table 3), no resultant significant reductions in biohydrogenation intermediates were seen (Table 3).

Remaining dry matter

Addition of the HP, T and HPT did not significantly alter dry matter degradation within the incubations compared to control incubations ($P = 0{\cdot}32$) and remaining dry matter expressed as a 100% of DM at 0 h was 94·4, 97·9, 84·7 and 88·7% after incubation for 6 h in the presence of Control, HP, T and HPT, respectively.

Discussion

We demonstrate that HP, T and HPT combinations cause some changes in the rumen microbiota and beneficial changes in rumen lipid metabolism *in vitro*. Nonetheless, although differences were visible upon HP treatment in dendrograms and canonical analysis of principal coordinates (CAP) ordination, PERMANOVA of HaeIII T-RFLP showed that the changes in the presence of HP were not significant, whilst T and HPT combinations did significantly alter the microbiota present within the incubations compared to control incubations. It may be true that only a few of the bacteria that change as a result of the addition of 'green odour' volatiles are actually able to biohydrogenate, thus, changes in the intensity of these key peaks relating to the biohydrogenating microbiota is enough to enable a significant change in biohydrogenation.

Table 3 Temporal proportional (% of total fatty acids) quantities of prevalent fatty acids within *in vitro* incubations in the absence of treatment (control) and presence of a hydroperoxide (Hydroperoxyoctadecatrienoic acid, HP, 200 μmol l^{-1}), terminal 'green odour' compounds (an equal combination of salicylic and jasmonic acid, which are commonly released due to plant damage, T, 200 μmol l^{-1}) and a combination of both (50 : 50; HP : T; HPT, 200 μmol l^{-1})

		Fatty acid									
Time (h)	Treatment	18:3n-3	C18:2n-6	CLA1	CLA *cis*-9, *trans*-11	C18:1 *cis*	C18:1 *trans*	C18:1 *trans*-10	C18:1 *trans*-11	C18:0	BOC
2	Control	51·59^{a}	13·63^{a}	0·38^{a}	0·13^{a}	3·57^{ab}	1·02^{a}	3·99^{a}	4·17^{a}	3·51^{a}	3·62^{a}
	HP	55·90^{b}	14·17^{b}	0·23^{b}	0·12^{a}	3·43^{c}	0·64^{b}	3·42^{b}	3·58^{b}	2·73^{b}	2·49^{b}
	T	50·60^{a}	13·13^{c}	0·37^{a}	0·13^{a}	3·46^{bc}	1·20^{a}	3·68^{c}	4·05^{a}	3·33^{ab}	2·50^{b}
	HPT	52·07^{a}	13·40^{ac}	0·32^{a}	0·11^{a}	3·60^{b}	1·05^{a}	3·69^{c}	3·93^{a}	3·74^{a}	3·24^{a}
	SED	0·82	0·13	0·02	0·00	0·05	0·09	0·07	0·14	0·30	0·20
	P-value	<0·001	<0·001	0·002	0·11	0·015	0·002	<0·001	0·018	0·024	0·001
4	Control	46·70^{a}	13·30^{a}	0·47^{a}	0·04^{a}	0·11^{ab}	1·69^{a}	0·08^{a}	1·14^{a}	4·01^{a}	4·15^{a}
	HP	55·87^{b}	14·52^{b}	0·24^{b}	0·02^{b}	0·12^{b}	0·54^{b}	0·02^{b}	0·34^{b}	2·66^{b}	2·20^{b}
	T	51·68^{c}	13·78^{c}	0·32^{c}	0·06^{c}	0·10^{ab}	1·00^{c}	0·04^{c}	0·66^{b}	3·49^{c}	3·18^{c}
	HPT	52·20^{c}	13·88^{c}	0·34^{c}	0·04^{a}	0·09^{a}	1·01^{c}	0·04^{c}	0·62^{b}	3·55^{c}	3·00^{b}
	SED	1·14	0·16	0·01	0·00	0·00	0·16	0·00	0·14	0·20	0·25
	P-value	<0·001	<0·001	<0·001	0·003	0·084	<0·001	<0·001	0·002	<0·001	<0·001
6	Control	44·90^{a}	13·49^{a}	0·62^{a}	0·64^{a}	0·11^{ab}	1·73^{a}	0·09^{a}	1·11^{a}	4·76^{a}	4·36^{a}
	HP	53·91^{b}	14·59^{b}	0·31^{b}	0·38^{b}	0·12^{ab}	0·70^{b}	0·03^{b}	0·43^{b}	2·91^{b}	2·51^{b}
	T	48·02^{ac}	13·78^{a}	0·53^{c}	0·75^{a}	0·10^{ab}	1·44^{a}	0·07^{bc}	0·95^{a}	4·14^{c}	3·61^{c}
	HPT	48·97^{c}	13·89^{a}	0·50^{c}	0·69^{a}	0·08^{a}	1·24^{a}	0·06^{bc}	0·81^{a}	4·08^{c}	3·18^{bc}
	SED	1·48	0·19	0·03	0·06	0·01	0·21	0·01	0·14	0·30	0·30
	P-value	0·002	0·002	<0·001	0·001	0·131	0·008	0·006	0·007	<0·001	0·002

SED, standard error of the mean; [1]sum of all isomers; CLA, conjugated linoleic acid; BOC, Branched and odd chain fatty acids. HP – hydroperoxides were produced from pure linolenic acid using maize lipoxygenase as described by Gardner and Grove (2001). T – jasmonic acid and salicylic acid were obtained from Sigma-Aldrich (St Louis, MO, USA).

Terminal restriction fragment length polymorphism lower peak number and Shannon's diversity indices in the presence of HP, T and HPT compared to control incubations in the absence of treatment suggest that the changes in the microbiota are due to the fact that these 'green odour' chemicals potentially have antimicrobial properties. MSPI T-RFLP data also support the fact that these 'green odour' chemicals are potentially antimicrobial, but due to the reduced diversity in the presence of this restriction enzyme, HaeIII T-RFLP data are considered more reliable. We have also previously demonstrated that MSP1 and Alu1 are not as good for obtaining diversity measures from the rumen microbiota (Huws *et al.* 2011). The superiority of HaeIII-based T-RFLP for obtaining better diversity measures has also been demonstrated for aquatic (Hartmann *et al.* 2005) and soil environments (Zhang *et al.* 2008). Irrespective, it is known that 'green odour' compounds function in cell signalling between plants (Creelman and Mullet 1997) and have potentially antimicrobial activity (Strobel *et al.* 2001; Cho *et al.* 2004). Strobel *et al.* (2001) demonstrated that the volatile 'green odour' compound 1-butanol, 3-methyl-, acetate, produced by an endophytic fungus, in particular had antimicrobial properties against a range of bacteria and fungi. Cho *et al.* (2004) demonstrated that the fatty acid oxidation volatiles (E,Z)-2,6-nonadienal (NDE) and (E)-2-nonenal (NE) isolated from cucumber had bactericidal activity against the human pathogens *Bacillus cereus*, *Escherichia coli* 0157:h7, *Listeria monocytogenes* and *Salmonella typhimurium*. In this study, we demonstrate that HP, T and thus the HPT combination potentially had antimicrobial properties against similar as yet uncultured bacterial genera, notably the genera: *Veillonellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Acetivibrio*, *Coprococcus*, *Clostridia*, *Anaerovibrio*, *Oscillibacter*, *Prevotella*, *Papillibacter* and *Leuconostoc*. Indeed, we previously found that fish oil also targeted as yet uncultured and unclassified *Clostridiales, Ruminococcaceae* and *Prevotella* similar to the bacterial genera likely targeted by HP and T (Huws *et al.* 2011) with a similar result of reducing biohydrogenation.

Despite the fact that HP's effects on the rumen microbiota were not significant, HP was a more potent inhibitor of biohydrogenation at all time intervals. Nonetheless, all treatments resulted in decreased biohydrogenation on average across the time points compared to control incubations in the absence of 'green odour' chemicals. It may be that bacteria became adapted to T somewhat at 6 h and/or they had transformed it to less toxic compound. Irrespective, this is the converse to what we previously found when cis-2-hexenol (C2H),

trans-2 decenal (T2H) and the hydroperoxide, 1,2-dimethylethyl hydroperoxide (1,2-DMEH) were added to *in vitro* incubations. C2H, T2H and 1,2-DMEH increased biohydrogenation resulting in decreased 18:3*n-3* and 18:2*n-6* and increased concentrations of *cis*-9, *trans*-11 CLA, 18:1 *trans*-10 and *trans*-11 and 18:0 (Lee *et al.* 2007). In the current study, more indicative chemicals released from damaged fresh grass appear to have a negative effect on biohydrogenation. These data in their entirety are suggestive that fatty oxidation products such as those found in the 'green odour' have differing effects on biohydrogenation, with long-chain HP and terminal acids (T) having a negative effect, whereas short-chain HP (1,2-DMEH) and leaf alcohols (C2H) have a positive effect.

In conclusion, addition of 'green odour' volatiles may be responsible in part to the better quality of milk produced in the summer in terms of their health beneficial fatty acid content. There is potential that these compounds can be added to ruminant feed supplements to improve the health benefits of milk all year round. To assess their potential to produce milk of equal health beneficial fatty acid content all year round, *in vivo* trials are required that would need to evaluate milk parameters as well as to ensure that ruminant efficiency is not detrimentally affected due to their potential antimicrobial nature.

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Conflict of Interest

The authors have no conflict of interest to declare.

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