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Sharpea and *Kandleria* are lactic acid producing rumen bacteria that do not change their fermentation products when co-cultured with a methanogen

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ABSTRACT

Sharpea and *Kandleria* are associated with rumen samples from low-methane-emitting sheep. Four strains of each genus were studied in culture, and the genomes of nine strains were analysed, to understand the physiology of these bacteria. All eight cultures grew equally well with D-glucose, D-fructose, D-galactose, cellobiose, and sucrose supplementation. D-Lactate was the major end product, with small amounts of the mixed acid fermentation products formate, acetate and ethanol. Genes encoding the enzymes necessary for this fermentation pattern were found in the genomes of four strains of *Sharpea* and five of *Kandleria*. Strains of *Sharpea* produced traces of hydrogen gas in pure culture, but strains of *Kandleria* did not. This was consistent with finding that *Sharpea*, but not *Kandleria*, genomes contained genes coding for hydrogenases. It was speculated that, in co-culture with a methanogen, *Sharpea* and *Kandleria* might change their fermentation pattern from a predominately homolactic to a predominately mixed acid fermentation, which would result in a decrease in lactate production and an increase in formation of acetate and perhaps ethanol. However, *Sharpea* and *Kandleria* did not change their fermentation products when co-cultured with *Methanobrevibacter olleyae*, a methanogen that can use both hydrogen and formate, and lactate remained the major end product. The results of this study therefore support a hypothesis that explains the link between lower methane yields and larger populations of *Sharpea* and *Kandleria* in the rumens of sheep.

1. Introduction

Methane (CH_4) emissions from sheep vary between individuals and these differences are stable and heritable [1,2]. The variation in emissions appears to be caused by differences in the animal [3–5], which result in different rumen microbial communities [6,7] which in turn result in differing ratios of the end products of rumen fermentation [8]. In sheep at least, there may be distinctive although not discrete bacterial assemblages associated with low and high CH_4 production [6]. *Sharpea* and *Kandleria* were identified as key bacteria associated with the S-type (*Sharpea*-enriched) bacterial community, one of the two low-CH_4 community types in rumen samples from sheep that differed in the amount of CH_4 formed per unit of feed consumed when these sheep were fed pelletized lucerne [6]. A metagenomic and metatranscriptomic

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study conducted by Kamke et al. [9] confirmed that the relative abundance of *Sharpea* spp. was greater in a group of low-CH_4 emitting sheep than in their high-CH_4 emitting flock mates. Furthermore, they observed that the D-lactate dehydrogenases of rumen bacteria associated with low-CH_4 emitting sheep were from *Sharpea* and *Kandleria*, and postulated that these bacteria produce lactate in the rumen. They also suggested that the lactate produced by these bacteria is further converted to butyrate by *Megasphaera* spp., resulting in lower CH_4 emissions from these sheep.

Little is known about *Sharpea* and *Kandleria*. They have been shown to produce lactate as their fermentation end products [10,11]. Some lactic acid bacteria modify their metabolism under certain environmental conditions, and shift from producing lactate to a mixed acid fermentation that also generates formate, acetate and ethanol [12,13], but this has not been studied in *Sharpea* and *Kandleria*.

In the rumen, formate and hydrogen (H_2) produced by bacteria are used by methanogens to form CH_4 [14]. It has been previously shown that low partial pressures of H_2 can stimulate thermodynamically more favourable fermentation pathways that result in more H_2 formation [15–17]. This is termed interspecies H_2 transfer. Interspecies formate transfer operates in a similar way, with formate use by methanogens or other formate-using microbes being postulated to favour increased formate production by bacteria that can do so [18–21]. The result is that, under rumen-like conditions where the concentrations of formate and dissolved H_2 are low [22,23], production of reduced fermentation products like lactate can be decreased in favour of acetate formation and increased production of H_2 or formate. This possibility was tested in the study described in this paper, by co-culturing *Sharpea* or *Kandleria* with a H_2- and formate-using methanogen. Our hypothesis was that, if the model suggested by Kamke et al. [9] is correct, then production of lactate by *Sharpea* and *Kandleria* should not be markedly affected by the presence of a methanogen using any H_2 or formate produced by the bacteria, consistent with their suggestion that *Sharpea* and *Kandleria* produce lactate in the rumen where H_2 and formate concentrations are naturally low. Conversely, if these bacteria produced less lactate and switched to a mixed acid fermentation when grown with methanogens, then the hypothesis of Kamke et al. [9] would not be supported.

2. Material and methods

2.1. Cultures

Stock cultures (frozen at −80 °C; AgResearch Rumen Microbiology collection) of four strains each of *Sharpea* and *Kandleria* were revived in 9 ml of RM02 medium supplemented with 0.5 ml of clarified rumen fluid [24], and grown in Hungate tubes with screw caps and butyl rubber stoppers (Bellco Glass, Vineland, NJ, USA), under a headspace of 100% CO_2. The clarified rumen fluid was amended with $CaCl_2$, $MgCl_2$ and vitamins [24], and 0.34% (w/v) D-glucose, 0.34% (w/v) D-cellobiose, 0.30% (w/v) D-xylose, 0.30% (w/v) L-arabinose, 0.88% (v/v) of sodium L-lactate syrup (50% in water), 2% (w/v) casamino acids, 2% (w/v) Bacto-peptone and 2% (w/v) yeast extract. Tubes were incubated statically at 39 °C. Once grown (optical density increase > 0.4, measured as described below), these cultures were subcultured every 24 h. The strains were *Sharpea azabuensis* strains RL1 (DSM 20406) from Bryant et al. [25], ST18^T (DSM 18934) from Morita et al. [26], and two isolates from the Hungate1000 Collection (KH1P5 and KH2P10 [27]), and *Kandleria vitulina* strains RL2^T (DSM 20405) from Bryant et al. [25], WCE2011 and MC3001 from Noel [28], and KHCV7 from the Hungate1000 Collection. ST18^T was isolated from horse faeces; all other strains were isolated from ruminants. Each of these strains was judged to be pure based on the cell type observed by microscopy, which matched the formal descriptions of *S. azabuensis* ST18^T [26] and *K. vitulina* RL2^T [11]. Sequencing the 16S rRNA genes of these cultures yielded the expected sequences without contaminating peaks in the electropherograms.

Methanobrevibacter olleyae strain 1H5-1P (DSM 16632) was purchased from the DSMZ (Braunschweig, Germany). *M. olleyae* was revived from frozen stocks, and grown in Hungate tubes in 9 ml of RM02 medium prepared under a headspace of 100% CO_2, supplemented with 60 mM sodium formate and 20 mM sodium acetate and 0.5 ml of clarified rumen fluid containing $CaCl_2$, $MgCl_2$ and vitamins (NoSubRFV [24]). A formate and acetate stock was sterilised by autoclaving under N_2 in a serum vial sealed with a butyl rubber stopper and an aluminium closure (Bellco Glass), so that addition of 0.5 ml of the stock to 9.5 ml of medium gave the desired final concentrations. After inoculation with 0.5 ml of a culture of *M. olleyae*, the tubes were pressurised with H_2:CO_2 (80:20 v/v) mixture to 1.4 bar overpressure, and incubated statically at 39 °C. Active cultures were maintained by weekly subculture.

2.2. Substrate utilisation tests

Single substrates were added to Hungate tubes containing 9 ml of RM02 medium and 0.5 ml of clarified rumen fluid containing Ca, Mg and vitamins, under a headspace of 100% CO_2. Sterile substrate stocks were prepared by filtering through 0.22 μm pore size Millex GP sterile filters (Millipore Corp., Bedford, MA, USA) via sterile syringes and needles into sterile N_2-flushed serum vials sealed with a butyl rubber stoppers and aluminium closures, so that addition of 0.5 ml of substrate stock to 9.5 ml of medium gave the desired final concentrations. The substrates tested and the initial concentrations are given in Table 1. To inoculate these, 0.5 ml of a culture of *Sharpea* or *Kandleria* was added to the tubes using CO_2-flushed disposable syringes and hypodermic needles. Each substrate treatment for each strain was conducted in triplicate with one additional uninoculated control tube, and growth compared to triplicate cultures with NoSubRFV but no added substrate. All tubes were incubated on an Orbitek XL orbiting platform (Infors HT, Basel, Switzerland) at 50 rpm and at 39 °C for 5 days, and the optical density at 600 nm was recorded every 24 h by inserting the tubes directly into a Spectronic 200 spectrophotometer (Thermo-Fisher Scientific Inc., Waltham, MA, USA). The spectrophotometer was set to zero absorbance using an uninoculated control tube.

2.3. Co-culture experiment

Co-culture experiments were carried out by growing *M. olleyae* (a hydrogen- and formate-utilising methanogen [29]) in combination with two strains each of *Sharpea* (ST18^T and KH1P5) or *Kandleria* (RL2^T and WCE2011). These experiments used 45-ml aliquots of RM02 medium supplemented with 2.5 ml of NoSubRFV in 120-ml serum vials under a headspace of 100% CO_2, in two separate sub-experiments (Fig. 1). All manipulations were made using CO_2-flushed disposable syringes and hypodermic needles. In the first variation of the experiment, *Sharpea* or *Kandleria* were first grown with 10 mM fructose (phase I) and *M. olleyae* was added later, after growth of the bacterium had ceased and any H_2 or formate had been formed. *M. olleyae* was then allowed to grow in this culture (phase II). To do this, *Sharpea* or *Kandleria* strains were grown for three days in 45 ml of RM02 medium supplemented with 2.5 ml NoSubRFV and final concentration of 10 mM fructose and then *M. olleyae* (2.5 ml of a late-log phase culture) was added. Samples for volatile fatty acid analysis were collected after inoculation of selected strains of *Sharpea* or *Kandleria* (Fig. 1, sample 1), after the bacteria had grown (Fig. 1, sample 2), then again when *M. olleyae*

Table 1
Substrates that supported growth of strains of *Sharpea* and *Kandleria*.[a]

Substrate	Conc.[b] (mM)	*Sharpea* strains				*Kandleria* strains			
		ST18^T	RL1	KH1P5	KH2P10	RL2^T	MC3001	WCE2011	KHCV7
DGlucose	4	+++[c]	+++	++	++	+++	+++	+	+++
DFructose	4	++	++	++	++	+++	+++	++	+++
DGalactose	4	+++	+++	+++	+++	+++	+	++	+++
Cellobiose	2	+++	+++	+++	+++	+++	+++	+	+++
Sucrose	2	+++	++	++	++	+++	+++	+	+++
Lactose	2	+++	++	++	+++	+++	+	−	+++
Raffinose	2	+++	+++	+++	+++	+++	−	−	−

[a] None of the strains grew with (initial substrate concentrations in mM) D-glucuronate (4), D-galacturonate (4), D-xylose (5), L-arabinose (5), L-rhamnose (5), glycerol (20), D-mannitol (4), L-glutamate (20), L-alanine (20), L-aspartate (20), citrate (20), fumarate (20), succinate (20), L-lactate (20), based on ΔOD < 0.1; all relative to controls without added substrate.
[b] Conc., initial concentration in growth medium.
[c] Optical density was measured at 600 nm. Symbols: +++, ΔOD > 0.4; ++, ΔOD 0.2 to 0.4; +, ΔOD 0.1 to 0.2; −, ΔOD < 0.1; all relative to controls without added substrate. The data are means of three replicate cultures.

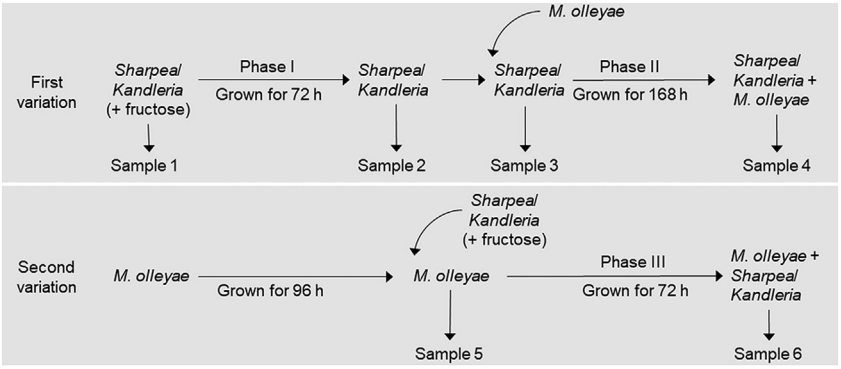

Fig. 1. Experimental design of co-culture experiments of *Sharpea* or *Kandleria* with *Methanobrevibacter olleyae*. Two variations of the experiments were conducted. In the first variation, cultures of *Sharpea* or *Kandleria* were allowed to grow and use all the fructose present (Phase I), before *M. olleyae* was added and allowed to grow (Phase II). In the second variation (phase III), *Sharpea* or *Kandleria* were inoculated into actively-growing cultures of *M. olleyae*. Samples for end product measurements were taken at different time points (denoted as samples 1 to 6). The incubation times were selected to ensure sufficient time for growth of *Sharpea* and *Kandleria* or *M. olleyae* in the first periods of 72 h or 96 h and for metabolism of substrates or products by the added cultures in the second periods of 168 h or 72 h. The time between samples 2 and 3 was 10 min.

was added (Fig. 1, sample 3) and lastly after one week of *M. olleyae* growth (Fig. 1, sample 4). In the second variation of the experiment, *M. olleyae* was grown first to establish an active population in late log phase, before *Sharpea* or *Kandleria* were added together with 10 mM fructose and allowed to grow (phase III). To do this, *M. olleyae* was grown for 4 days in 45 ml of RM02 medium supplemented 2.5 ml of NoSubRFV and with final concentrations of 60 mM sodium formate and 20 mM sodium acetate, and the vials were pressurised with H_2:CO_2 (80:20 v/v) mixture to 1.4 bar overpressure. Once grown, the vial headspaces were flushed with 100% CO_2 using a 0.22 μm pore size filter with a hypodermic needle to introduce the gas and a second hypodermic needle as a gas outlet. *Sharpea* or *Kandleria* (0.5 ml of a late-log phase culture) was then added to the vials together with fructose to a concentration of 10 mM. Samples were collected immediately after inoculation of *Sharpea* or *Kandleria* (Fig. 1, sample 5) and again after 72 h of growth of the bacteria with *M. olleyae* (Fig. 1, sample 6). All incubations were at 39 °C, and the vials were shaken on an Orbitek XL orbiting platform at 50 rpm.

2.4. End product analyses

Samples (2 ml) were collected from substrate utilisation and co-culture experiments for end product analyses. Samples were centrifuged at 5000 g for 5 min and supernatant fractions were collected and filtered through cellulose-free sterile syringe filters (0.22 μm pore size; Millipore) and stored at −20 °C until analysed. Substrate and product concentrations were measured using high-performance liquid chromatography (LC10AVP, Shimadzu Scientific Instruments, Columbia, MD, USA). The column was an Aminex HPX-87H column (dimensions 300 × 7.8 mm; Bio-Rad, Miami, FL, USA), the temperature was 45 °C, 5 mM sulfuric acid was the mobile phase at a flow rate of 0.8 ml per minute, and quantification was made using a RID 10A refractive index detector (Shimadzu Scientific Instruments). The injection volume was 50 μL. Standards containing fructose, lactate, succinate, acetate, formate, propionate, butyrate and ethanol were prepared over a concentration range of 2.5 mM–20 mM, and used to prepare standard calibrations for converting the detector outputs to concentrations. The calibrations showed linear relationships to 20 mM.

H_2 production was assessed by gas chromatography. Samples (1 ml) of culture headspace, at the pressure in the culture tube, were collected using disposable plastic syringes fitted with Mininert Luer-tip syringe valves (Hamilton, Reno, NV, USA) and injected into an Aerograph 660 gas chromatograph (Varian Associates, Palo Alto, CA, USA) fitted with a Porapak Q80/100 mesh column (Waters Corporation, Milford, MA, USA) and a thermal conductivity detector. The column was operated at room temperature with nitrogen gas (N_2) as the carrier gas at 14 cm^3/min. Standards of H_2 at 0.05% (v/v) and 1% (v/v) in N_2 were used for calibration (alpha standards; BOC Gas, Palmerston North, New Zealand). Dissolved H_2 concentrations were calculated from the headspace concentrations using Ostwald coefficients tabulated by Wilhelm et al. [30] and molar gas volumes from Battino [31], assuming that the headspace gases

were in equilibrium with the dissolved gases. The culture tubes were incubated on an orbital platform to aid this equilibration (see above). The detection limit was about 20 nM dissolved H_2.

The D-Lactic Acid Assay Kit and L-Lactic Acid Assay Kit (Megazyme Inc., Bray, Ireland) were used for measurements of D- and L-lactate concentrations, respectively. All samples were diluted to yield a lactic acid concentration of 0.03–0.30 g/L, the linear range of the assay. The microplate assay procedure was performed according to manufacturer's instructions with a 224 μL reaction volume.

The percentage of each of the organic fermentation end products was calculated for all eight strains tested, and analysis of variance (ANOVA) was carried out to examine the significance of genus (*Kandleria* and *Sharpea*) and strain differences (four per genus). In the experiment in which four strains of bacteria were each grown with *M. olleyae*, the ratio of the end products lactate, formate, acetate and ethanol to the amount of fructose used was calculated and ANOVA was carried out on response variables to examine the significance of genus (*Kandleria* and *Sharpea*), strain differences (2 per genus) and phase of the experiment (I, II, III). The analysis was conducted using GenStat [32].

2.5. Genome based study

Sharpea and *Kandleria* genomes used in this study were sequenced as a part of the Hungate1000 project [27] by the DOE Joint Genome Institute (JGI GOLD Study ID Gs0033970). Genomes of four *Sharpea* strains (ST18^T = IMG Genome ID 2561511132, RL1 = IMG Genome ID 2606217806, KH1P5 = IMG Genome ID 2606217758, and KH2P10 = IMG Genome ID 2606217759) and five *Kandleria* strains (RL2^T = IMG Genome ID 2561511092, WCE2011 = IMG Genome ID 2558860130, MC3001 = IMG Genome ID 2558309014, KH4T7 = IMG Genome ID 2654588124, and S3b = IMG Genome ID 2606217761) were used in this study to deduce the fermentation pathways of these bacteria. Genes from *Sharpea* and *Kandleria* genomes were first mapped to KEGG pathways using the integrated genome and metagenome comparative data analysis system (IMG/M) [33] to obtain initial evidence for the presence or absence of particular genes. Reviewed amino acid sequences of queried genes were collected from UniProtKB/Swiss-Prot [34] and analysed by BLAST [35] against the *Sharpea* and *Kandleria* genomes to confirm the presence or absence of individual genes. Genes coding for enzymes involved in transport system were identified by BLAST of *Sharpea* and *Kandleria* genomes in the TransportDB 2.0 database [36]. Genes of putative hydrogenases were also confirmed by BLAST against a custom database of hydrogenases [37].

3. Results and discussion

3.1. Substrates that support growth of Sharpea and Kandleria

Four strains each of *Sharpea* and *Kandleria* were used to identify substrates that supported growth and used to determine fermentation products. We selected substrates that could be available to these bacteria in the rumen and that were soluble and could be readily measured in culture supernatants. The affiliations of these strains were first confirmed by phylogenetic tree reconstruction based on almost full-length 16S rRNA gene sequences (Supplementary Fig. 1). All eight cultures were inoculated into growth medium supplemented with different growth substrates. Of the 21 substrates tested, only D-glucose, D-fructose, D-galactose, cellobiose, and sucrose supported growth of all eight strains of *Sharpea* and *Kandleria* (Table 1). However, the amount of growth obtained with strain WCE2011, as determined by optical density of the cultures, was generally less than with the other strains. Lactose supported growth of seven of the strains, and raffinose of five. None of the tested strains were able to ferment xylose, arabinose, glycogen, and glycerol. None of the strains grew with some common organic and amino acids (Table 1). These findings are similar to, and extend, those of Sharpe et al. [10] for strains RL1 and RL2^T.

3.2. End products of Sharpea and Kandleria

The end products of fermentation by the eight strains of *Sharpea* and *Kandleria* in pure culture were determined by growing strains in medium containing 10 mM D-fructose. All strains produced lactate as the major end product (~75% of total end product; Table 2), which was almost exclusively D-lactate. Small amounts of formate, acetate and ethanol were also produced, but propionate, succinate and butyrate were not. There were differences in the proportions of the different products formed by the eight strains. No unidentified peaks were observed in the chromatograms. Lactate production was reported by Sharpe et al. [10] and Salvetti et al. [11] for strains that were eventually classified as *Sharpea* and *Kandleria*, but the other products have not been previously reported.

Strains of *Sharpea* produced traces of H_2, but the maximal concentrations detected were only 1360 ppm in the culture headspace, equivalent to about 85 μmol produced per litre of culture and 1.1 μM dissolved H_2. This was similar when no fructose was added (maximally 1.0 μM dissolved H_2), and so the small amount formed appeared to originate from medium components other than fructose. Other products were formed in multiple millimoles per litre. H_2 was therefore not a major product of fermentation, with final concentrations being three orders of magnitude less than formate. Dissolved H_2 concentrations in the cultures of *Kandleria* strains were even smaller, all less than 25 nM, close to the detection limit which was about 20 nM and the same as in uninoculated medium. This suggests that *Kandleria* does not form H_2.

3.3. Is the fermentation pattern of Sharpea and Kandleria influenced by a methanogen?

It was clear that *Sharpea* and *Kandleria* produced lactate as their major end product in pure culture. It has been reported that homolactate fermenters (Fig. 2A) can change their behaviour and perform a mixed acid fermentation (Fig. 2B) to varying extents, depending on the culture conditions [12,13,38]. The production of small amounts of acetate, formate and ethanol by *Sharpea* and *Kandleria* indicate that there is a minor role for a mixed acid fermentation in their metabolism (Fig. 2C). Because small amounts of acetate, formate and ethanol were being produced, we tested whether the fermentation products of *Sharpea* and *Kandleria* were influenced by the presence of a methanogen, to determine if formate and H_2 use by the methanogen could increase the flow of products to the mixed acid pathway.

M. olleyae was chosen for co-culture experiments with *Sharpea* or *Kandleria*, as it can use both H_2 and formate as electron donors [29]. The experiment was conducted as shown in Fig. 1. In phase I of this experiment, pure cultures of *Sharpea* and *Kandleria* were allowed to grow using fructose, before *M. olleyae* was added and allowed to grow (phase II). It was hypothesised that there should be no change in the bacterial fermentation products after *M. olleyae* was added compared to the products detected just before the addition of the methanogen, because the bacteria would have used all the fructose prior to the addition of the methanogens. Only the formate produced by the bacteria could be used by the methanogen during phase II, and there would be no opportunity for the activity of *M. olleyae* to modify the fermentation patterns of the bacteria. In a second variation of this experiment, *M. olleyae* was allowed to

Table 2
End products of fructose fermentation by *Sharpea* and *Kandleria*.

Genus	Strain	Fructose remaining[a,b] (mM)	Products formed (mM)[b]			
			Lactate[c,d]	Formate[e]	Acetate[f]	Ethanol[g]
Sharpea	ST18^T	0.01	17.96	2.79	1.92	0.69
	RL1	0.01	15.94	1.41	0.76	<0.01
	KH1P5	0.01	15.05	1.32	0.68	0.12
	KH2P10	0.06	15.56	1.40	1.31	0.60
Kandleria	RL2^T	0.05	15.44	1.57	0.78	1.03
	MC3001	<0.01	14.07	1.98	1.09	0.42
	WCE2011	<0.01	15.30[h]	1.95	0.94	0.40
	KHCV7	0.05	16.14	1.90	1.07	0.17

[a] Initial fructose concentration was 10 mM.
[b] All data are means of three replicate cultures.
[c] Only D-lactate detected, unless noted otherwise.
[d] p-values in ANOVA, 0.228 between genera, 0.011 for strains.
[e] p-values in ANOVA, 0.353 between genera, 0.125 for strains.
[f] p-values in ANOVA, 0.028 between genera, 0.001 for strains.
[g] p-values in ANOVA, 0.079 between genera, 0.008 for strains.
[h] Mainly D-lactate, <7% L-lactate.

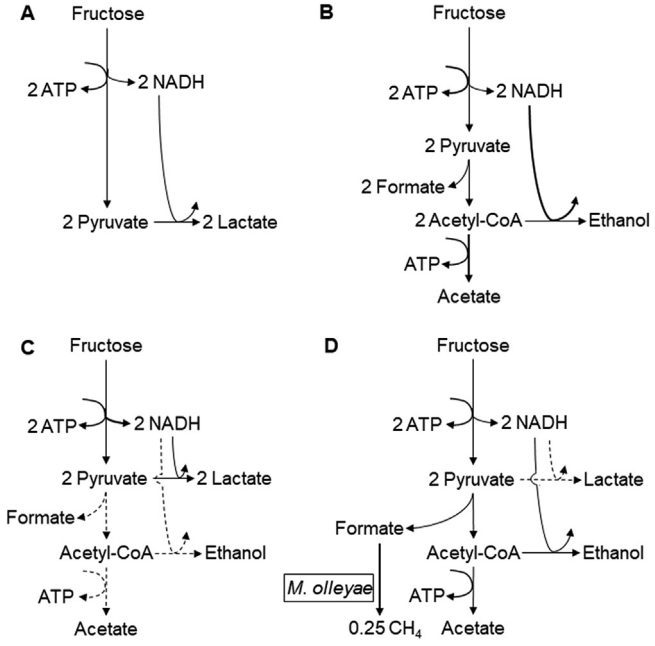

Fig. 2. Possible fermentation schemes. A) Homolactic fermentation. B) Formate-acetate-ethanol mixed acid fermentation. C) Predominantly lactate formation by *Sharpea* and *Kandleria* with a minor role for formate, acetate and ethanol formation. D) Predicted change in end-product formation in the case of inter-species formate transfer or change in fermentation pattern from homolactic to mixed acid fermentation in the presence of *M. olleyae*. Dashed arrows indicate less active pathways. Note that the schemes in panels C and D are not balanced.

grow to late log phase before adding fructose and inocula of *Sharpea* and *Kandleria*. In this case, the activity of *M. olleyae* during the subsequent growth of the bacteria (in phase III) could potentially modify the fermentation pattern of the bacteria. If *Sharpea* and *Kandleria* performed a mixed acid fermentation that was influenced by end-product use by the methanogen, then in the presence of *M. olleyae* the final lactate concentration would decrease, and the acetate and perhaps ethanol concentrations would increase (Fig. 2D). This would lead to additional ATP formation at the acetate kinase step (Fig. 2D), and so could be advantageous to the bacteria. However, this did not happen. The dominance of lactate as the major end product did not change when *Sharpea* and *Kandleria* were grown in the presence of *M. olleyae* compared to when there was no methanogen present, or when the methanogen was added after the bacteria had grown (Table 3). The amount of ethanol formed also was not significantly different between the phases of this experiment. Formate was used by *M. olleyae* in the co-cultures with the bacteria, and therefore the amounts were significantly different between the phases of the experiment. The amounts of acetate also varied, but this was most likely due to the acetate added with the methanogen inoculum (Table 3). Overall, it appeared that in co-culture with the methanogen, *Sharpea* and *Kandleria* kept performing a predominantly homolactic acid fermentation (Fig. 2C) and did not change their metabolism so that more mixed acid fermentation products were formed.

3.4. Genomic prediction of end product formation by Sharpea and Kandleria

Genes coding for enzymes involved in sugar transport and fermentation were searched for in *Sharpea* and *Kandleria* genomes (Supplementary Table 1). BLAST searches of *Sharpea* and *Kandleria* genomes against TransportDB suggested that both genera are able to transport sugars using the phosphotransferase system (PTS). All genomes contained one gene coding for enzyme I of the PTS for sugar uptake, and three genes coding for HPr protein, which accepts a phosphoryl group from phosphoenolpyruvate via enzyme I and phosphorylates enzyme II. Enzyme II of the PTS is made up of 4 domains that are contained in a multi-subunit complex, where each subunit contains one of more domains [39]. The *Sharpea* genomes each contained 43 to 49 genes coding for different subunits required for functional enzyme II, in varying combinations of domains in each subunit. The *Kandleria* genomes contained 20 to 33 genes coding for subunits of enzyme II. In both genera, these were predicted to transport a range of sugars: cellobiose, fructose, mannose, glucose, maltose and *N*-acetylglucosamine.

It appeared that *Sharpea* and *Kandleria* can also transport sugars using ABC transporters (Supplementary Table 1). Each of the *Sharpea* and *Kandleria* genomes contained 3 to 5 genes coding for possible sugar-binding proteins that are predicted to be part of ABC transporters [40]. The other components of ABC transporters were also coded for in all genomes, including 4 to 8 genes in each genome predicted to code for the membrane subunits and one gene for ATP-binding proteins that were predicted to be involved in sugar transport. All the genomes also encoded one or two genes for glucokinase, fructokinase, and galactokinase, which would phosphorylate the transported sugars for further metabolism.

Table 3
Changes in concentrations of substrate and fermentation products of *Sharpea* or *Kandleria* with and without *M. olleyae*. See Fig. 1 for experimental design.

Phases of experiment (samples for calculations)	Strain	Substrates and products (mM)[a]				
		Fructose	Lactate[b]	Formate[b]	Acetate[b]	Ethanol[b]
I. Pure culture growth of *Sharpea/Kandleria* (2–1)	ST18^T	−10.96	18.00	1.98	1.13	0.29
	KH1P5	−12.23	20.72	1.48	1.30	0.29
	WCE2011	−10.05	15.63	1.96	1.01	0.13
	RL2^T	−9.82	15.61	1.63	1.01	0.23
II. *M. olleyae* added to grown cultures of *Sharpea/Kandleria* (4–1)	ST18^T	−11.01	17.76	−0.01	2.27[d]	0.23
	KH1P5	−12.27	19.99	0.00	2.56[d]	0.20
	WCE2011	−10.05	15.47	0.51	2.09[d]	0.07
	RL2^T	−9.82	15.82	0.00	2.24[d]	0.19
III. *Sharpea/Kandleria* added to grown culture of *M. olleyae* (6–5)	ST18^T	−9.72	15.21	−0.93[c]	3.13	0.20
	KH1P5	−10.77	18.53	−0.22[c]	1.24	0.14
	WCE2011	−9.87	17.76	−0.95[c]	1.56	0.06
	RL2^T	−8.96	15.55	−0.64[c]	1.46	0.07

[a] Negative values indicate a decrease in concentration. All data are the means of three replicate cultures. Changes in concentration were calculated from the differences at different sampling points (indicated by the numbers 1 to 6; see Fig. 1 for details).
[b] p-values in ANOVA for differences between phases of the experiment: lactate, 0.088; formate, < 0.01; acetate, < 0.001; ethanol, 0.496.
[c] 0.64–1.07 mM formate remaining in the methanogen culture and introduced with the inoculum when *Sharpea* and *Kandleria* strains were added, which was then used by *M. olleyae* together with any formate formed during fructose fermentation. All final formate concentrations were below the detection limit, 0.02 mM.
[d] 0.77–1.16 mM residual acetate added when *M. olleyae* was inoculated into cultures with *Sharpea* or *Kandleria* strains (sample 3 in Fig. 1).

Once the sugars are imported and phosphorylated, it appears that these bacteria use a standard glycolytic pathway to convert hexoses to two pyruvate molecules (Supplementary Table 1). In this process, one molecule of ATP is used (at the step converting fructose-6-phosphate to fructose-1,6-bisphosphate) and three ATP are produced (two at the 1,3-bisphosphoglycerate to 3-phosphoglycerate step and one at the phosphoenolpyruvate to pyruvate step, assuming that one phosphoenolpyruvate is used in the PTS step), resulting in a net gain of two ATP per hexose. If sugars are imported using ABC transporters, then two ATP are used in the import and initial phosphorylation steps, but four ATP are formed later in the pathway, also giving a yield of two ATP per hexose. Genes coding for enzymes involved in the pentose phosphate pathway (PPP) and the Entner-Doudoroff pathway of sugar fermentation [41,42] were also searched for in the *Sharpea* and *Kandleria* genomes. Genes for key enzymes in the initial oxidative branch of the PPP were absent, suggesting that this pathway is incomplete and does not take part in sugar fermentation. However, a partial PPP may be involved in formation of precursors (D-ribose 5-phosphate, D-ribulose 5-phosphate, D-xylulose 5-phosphate and D-erythrose 4-phosphate) required for a number of biosynthetic pathways. The Entner-Doudoroff pathway was also absent in *Sharpea* and *Kandleria*, as genes coding for key enzymes (6-phosphogluconate dehydratase and 2-keto-3-deoxygluconate 6-phosphate aldolase) were missing in all analysed genomes. The presence of a phosphoglucomutase suggests that these species may store glycogen, but this remains to be studied.

It was interesting to find that, even though only D-lactate was detected in the *in vitro* fermentation experiments with 7 of the 8 strains, the genomes of *Sharpea* and *Kandleria* appear to contain genes for both D- and L-lactate dehydrogenases (Supplementary Table 1). This was found using a custom BLAST against the UniProtKB/Swiss-Prot database. The predicted protein sequences of the putative *Sharpea* and *Kandleria* D-lactate dehydrogenases showed ≥44.1% identity with a reviewed D-lactate dehydrogenase from *Escherichia coli* (Swiss-Prot accession number P52643), whereas predicted protein sequences of the putative L-lactate dehydrogenases were ≥46.4% identical to a reviewed L-lactate dehydrogenase from *Bacillus cereus* (Swiss-Prot accession number Q815X8). Genes coding for lactate isomerase were not found. Lactate permease was coded for in all nine genomes of *Sharpea* and *Kandleria*, and presumably is responsible for lactate transport out of the cell.

Production of formate, acetate and ethanol *in vitro* was supported by the presence of genes (Supplementary Table 1) involved in pathways forming these products [43]. Both *Sharpea* and *Kandleria* possess genes for pyruvate-ferredoxin/flavodoxin oxidoreductase, phosphoacetyl transferase and acetate kinase, which would form acetate. Genes for pyruvate formate lyase, involved in formation of acetyl-CoA and formate, were also present in all the analysed genomes, indicating formate production and also acetyl-CoA formation that could lead to acetate and ethanol formation. Formate dehydrogenase genes were absent, indicating that formate is produced as a final end product that is not further converted to H_2 and CO_2. Production of ethanol by *Sharpea* and *Kandleria* strains was supported by the presence of genes that encode acetaldehyde dehydrogenase and alcohol dehydrogenase, which act to form ethanol from acetyl-CoA. Genes coding for key enzymes involved in propionate [44] and butyrate [45] formation were absent from all *Sharpea* and *Kandleria* genomes, consistent with the finding that these bacteria did not form propionate or butyrate. The marker genes for propionate formation, coding for lactoyl-CoA dehydratase (acrylate pathway), propionaldehyde dehydrogenase (propanediol pathway), and methylmalonyl-CoA decarboxylase (randomising succinate pathway), were not found. The marker genes for butyrate formation, coding for acetyl-CoA acetyltransferase, hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, butyryl-CoA dehydrogenase, phosphotransbutyrylase and butyrate kinase via acryl pathway were also not found in any of the analysed genomes (Supplementary Table 1).

Subunits of a [FeFe] group A2 [37] hydrogenase, which contains two subunits (HydA and GltA), were present in all *Sharpea* genomes but were absent from the *Kandleria* genomes. The predicted protein sequences of the catalytic (HydA) subunit had > 58.48% identity with a homologue from *Olsenella profusa* (GenBank accession number WP_021726228) while the NADH-dependent subunit (GltA) had > 35.50% identity to a homologue in *Haloferax volcanii* (GenBank accession number WP_004044456). Additionally, a group B1/B3 monomeric [FeFe] hydrogenase may be present in all *Sharpea* genomes but absent from all *Kandleria*. This was supported by BLAST searches of the predicted amino acid sequences against the Greening et al. [37] database, where > 34.7% identity was found with HydM of *Bacteroides intestinalis* (GenBank accession number WP_007665069).

Genes potentially coding for a NAD(P)-dependent iron-only hydrogenase catalytic subunit and for a NAD(P)-dependent iron-only hydrogenase diaphorase component flavoprotein were found in the *Kandleria* genomes by mapping to KEGG pathways. However, no match was found using BLAST against the Greening et al. [37] database. Also, BLAST searches of the putative NAD(P)-dependent iron-only hydrogenase catalytic subunits against the UniProtKB/Swiss-Prot database resulted in matches to other proteins, with the greatest identities (34.42–61.04%) to a MreB-like protein (GenBank accession number WP_003227776). BLAST searches of the potential NAD(P)-dependent iron-only hydrogenase diaphorase component flavoprotein resulted in matches with putative dipeptidases (GenBank accession number NP_179247), with 35.44–36.41% identity. It is therefore likely that *Kandleria* lacks hydrogenases. The lack of genes encoding hydrogenases in the *Kandleria* genomes, and their presence in the *Sharpea* genomes is consistent with production of H_2 in our culture-based experiments, and suggest that hydrogenases and H_2 production are characteristics that differentiate these two genera.

3.5. Conclusions

In vitro studies with *Sharpea* and *Kandleria* confirmed previous findings [10,11] that these behave like classical lactic acid bacteria that produce lactate as their major end product. Small amounts of acetate, formate and ethanol were produced from fructose, suggesting that they might perform a mixed acid fermentation in certain circumstances, and this is a new observation for these genera. Production of formate also raised the possibility that, in the presence of a H_2- or formate-using methanogen, these bacteria might change their metabolism to producing less lactate and more acetate and ethanol. However, co-culture experiments with *M. olleyae* (a methanogen that can use H_2 and formate) appeared to refute this hypothesis, as lactate remained the major end product. The small amounts of acetate, formate and ethanol produced in both the pure cultures and co-cultures are the products of a background mixed acid fermentation that may yield acetyl-CoA, reducing the potential for cell synthesis, or for some other purpose. This is different to other fermenting bacteria that change the ratios of their end products in the presence of methanogens [15,17,19,46].

The findings of this study are in agreement with the proposal by Kamke et al. [9] that, in the complex rumen system where methanogens are active, *Sharpea* and *Kandleria* produce mainly lactate. Lactate formation, with further conversion of lactate to butyrate by the action of *Megasphaera* spp., results in less H_2 formation than the classical direct fermentation of carbohydrates to butyrate by bacteria such as members of the family *Ruminococcaceae*. This is proposed to be the reason why less CH_4 is formed in the rumen of sheep with greater populations of *Sharpea* and *Kandleria* [9].

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anaerobe.2018.07.008.

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