Rapid changes in key ruminal microbial populations during the induction of and recovery from diet-induced milk fat depression in dairy cows

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Abstract

The ruminant provides a powerful model for understanding the temporal dynamics of gastrointestinal microbial communities. Diet-induced milk fat depression (MFD) in the dairy cow is caused by rumen-derived bioactive fatty acids, and is commonly attributed to the changes in the microbial population. The aim of the present study was to determine the changes occurring in nine ruminal bacterial taxa with well-characterised functions, and abundance of total fungi, ciliate protozoa and bacteria during the induction of and recovery from MFD. Interactions between treatment and time were observed for ten of the twelve populations. The total number of both fungi and ciliate protozoa decreased rapidly (days 4 and 8, respectively) by more than 90 % during the induction period and increased during the recovery period. The abundance of *Streptococcus bovis* (amylolytic) peaked at 350 % of control levels on day 4 of induction and rapidly decreased during the recovery period. The abundance of *Prevotella bryantii* (amylolytic) decreased by 66 % from day 8 to 20 of the induction period and increased to the control levels on day 12 of the recovery period. The abundance of *Megasphaera elsdenii* and *Selenomonas ruminantium* (lactate-utilising bacteria) increased progressively until day 12 of induction (> 170 %) and decreased during the recovery period. The abundance of *Fibrobacter succinogenes* (fibrolytic) decreased by 97 % on day 4 of induction and increased progressively to an equal extent during the recovery period, although smaller changes were observed for other fibrolytic bacteria. The abundance of the *Butyrivibrio fibrisolvens/Pseudobutyrivibrio* group decreased progressively during the induction period and increased during the recovery period, whereas the abundance of *Butyrivibrio hungatei* was not affected by treatment. Responsive taxa were modified rapidly, with the majority of changes occurring within 8 d and their time course was similar to the time course of the induction of MFD, demonstrating a strong correlation between changes in ruminal microbial populations and MFD.

Key words: Milk fat depression: Rumen microbes: Dairy cows: Conjugated linoleic acid

Diet-induced milk fat depression (MFD) is caused by the inhibition of milk fat synthesis by bioactive fatty acids (FA) synthesised by rumen microbes, and is a well-studied example of the interaction between dietary nutrients, the gastrointestinal microbiome and tissue physiology. Rumen microbes perform a wide range of functions and are classically grouped within niches based on their predominant substrate or enzyme activity. Moreover, rumen microbes biohydrogenate unsaturated FA, resulting in the formation of *trans* isomers as intermediates. The rate, extent and pathways of biohydrogenation (BH) are commonly attributed to the microbial population present in the rumen. Previous investigations quantifying ruminal microbial populations have been conducted after diet adaptation periods; however, the time course of adaptation to a new diet is not well characterised.

Importantly, investigation of the time course is essential to establish the potential for the changes in the microbial population as a primary mechanism in diet-induced milk fat depression, rather than simply as a secondary adaptation.

Milk FA are a sensitive indicator of absorbed FA profile, as 85 % of preformed FA originate directly from intestinal absorption in cows in positive energy balance[1]. Shingfield *et al.*[2] and Rico & Harvatine[3] reported the time course of the changes in milk FA profile when cows were switched from a control diet to a diet that resulted in MFD because of high diet fermentability and high PUFA concentration. During the transition period, a biphasic response in BH intermediates was observed[3]. The rate or capacity of the normal rumen BH pathway was first decreased, causing a 2- and 3-fold increase in *trans*-11 18 : 1 and *cis*-9, *trans*-11-conjugated

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linoleic acid (CLA) on days 3 and 5, respectively. After day 5, these intermediates decreased rapidly. At the same time, there was a progressive increase in the alternative BH pathway that became predominant after 9 d as indicated by an increase in the production of *trans*-10 18 : 1 and *trans*-10, *cis*-12-CLA. Similar temporal shifts in microbial populations are expected to be the driver of these metabolic changes.

Rumen microbes are sensitive to dietary risk factors for MFD including diet fermentability and unsaturated FA concentration, low ruminal pH, and monensin. For example, Weimer *et al.*[4] reported broad changes in ruminal bacterial communities when feeding high-starch and monensin-supplemented diets using automated ribosomal intergenic spacer analysis, and others have reported changes in specific taxa by real-time quantitative PCR[5–7]. Furthermore, Rico *et al.*[8] reported that inoculation of MFD cows with rumen digesta from cows fed a high-fibre and low-PUFA diet slightly accelerated the recovery of normal ruminal BH intermediates in MFD cows, thus suggesting that re-establishment of a normal ruminal microbial population may limit the rate of recovery from MFD.

The objective of the present study was to determine the time course of the shift in the rumen microbiome during dietary changes by investigating selected microbial taxa with well-characterised functions using quantitative PCR, which is a sensitive method to detect changes[9]. Similarly to Mullins *et al.*[7], we propose that the selected taxa would provide a proxy of general changes in the microbial ecosystem. The classic culturable bacterial species represent only a small fraction of the total rumen microbiome[6]; however, specific well-characterised bacterial species are expected to represent the niche they occupy in the rumen and to demonstrate the temporal adaptation of the rumen. Additionally, the ruminant is an interesting model for studying the gastrointestinal microbiome, as cannulation allows direct sampling of the most active fermentation compartment. We hypothesised that rumen microbes would rapidly adapt after a dietary switch, and that the dynamics of rumen taxa would correlate with changes in the profile of milk *trans*-FA.

Materials and methods

Experimental design

All experimental procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. All cows were housed in a tie-stall barn located at the Pennsylvania State University's Dairy Production Research and Teaching Center, and fed individually once daily (08.00 hours) at 110 % of expected intake.

A total of eight ruminally cannulated cows were used to investigate the time course of the induction of and recovery from MFD in a replicated design with three periods of 21 d (one cow failed to reduce milk fat and was not included in the experiment). A detailed description of the experimental design and treatments has been reported previously[3]. Briefly, in each treatment sequence, induction of MFD followed the control period, and recovery from MFD followed the induction period (Table 1). A pre-trial period was necessary

Table 1. Treatment assignment of a repeated design to study the induction of and recovery from diet-induced milk fat depression

Assignment	Pre-experiment	Period 1	Period 2	Period 3
1	Control	Control	Induction	Recovery
2	Induction	Recovery	Control	Induction
3	Control	Induction	Recovery	Control

to provide an induction period before recovery in period 1 for treatment sequence 2. Induction of milk fat depression was achieved by feeding cows a low-fibre, high-PUFA diet (29·5 % neutral-detergent fibre (NDF), 27 % starch, 5·5 % FA and 3·7 % PUFA; DM basis) for a period of 21 d. Following the induction of MFD, dairy cows were switched to a high-fibre, low-PUFA diet (36·9 % NDF, 18 % starch, 2·6 % FA and 1·1 % PUFA) to observe recovery of milk fat for 21 d.

Sampling procedures

Whole rumen digesta samples were collected at 16.00 hours on days 0, 4, 8, 12 and 20 of each experimental period. The sample was collected approximately 8 h after feeding, and represents the high intake and rapid fermentation period of the day. Digesta samples were collected from five different locations of the rumen (cranial dorsal, cranial ventral, central, caudal dorsal and caudal ventral), composited, subsampled (approximately 250 g) and stored at −20°C. The samples were freeze-dried (Ultra 35-XL; Virtis Company, Inc.) and ground in a coffee grinder (Model 80 335; Hamilton Beach Brands, Inc.) for 60 s before DNA extraction. Importantly, the procedures used herein provided whole digesta samples representing the microbial populations in both the liquid and the solid fractions (see Mullins *et al.*[7]).

DNA extraction and quantitative PCR analysis

DNA was isolated once for each cow at each time point using a commercially available kit (QIAamp DNA Stool Mini Kit; Qiagen Sciences) with modifications similar to those proposed by Yu & Morrison[10]. Briefly, approximately 220 mg of freeze-dried and ground digesta were homogenised for 5 min with 1·2 ml lysis buffer and 0·4 g of 0·1 mm sterile zirconia beads using a bench-top vortex equipped with a MO BIO Vortex-Genie 2 adapter (MO BIO Laboratories). A mixture of 0·5 and 0·1 mm beads, as proposed by Yu & Morrison[10], was tested, but did not improve the yield. After bead-beating, the samples were incubated twice at 95°C for 15 min with vortexing every 5 min and bead-beating between the incubation periods. Lastly, DNA was purified by column extraction, and DNA concentration was determined by spectroscopy (NanoDrop ND-1000 Spectrophotometer; Nano-Drop Technologies). The repeated bead-beating and the column purification in the extraction method provided high yield and quality of DNA[10,11]. The relative abundances of lactate-utilising bacteria (*Megasphaera elsdenii* and *Selenomonas ruminantium*), amylolytic bacteria (*Streptococcus bovis* and *Prevotella bryantii*), fibrolytic bacteria (*Ruminococcus albus*, *Fibrobacter succinogenes*, and *Prevotella ruminicola*), bacteria involved in BH (*trans*-11 18 : 1 producers, the *Butyrivibrio*

fibrisolvens/Pseudobutyrivibrio group and *Butyrivibrio hungatei*), anaerobic fungi (Neocallimastigales) and ciliate protozoa were determined in triplicate using real-time quantitative PCR and previously validated primers and conditions (400 nM of each primer; see online Supplementary Table S1). Reactions were performed using a commercial mix (PerfeCTa SYBR Green SuperMix with ROX; Quanta Biosciences), and amplification fluorescence was measured (Applied Biosystems 7900 HT Fast Real-Time PCR System; Life Technologies). Primer specificity was evaluated by melting curve analysis, and efficiency (E) was calculated as $E = 10^{-1/\text{slope of the standard curve}}$. Efficiency values ranged between 1·86 and 2·27 (mean 2·04). Abundance of bacterial species are expressed as a percentage of total bacteria, calculated as $E(\text{UB})^{C_t(\text{UB})}/E(\text{TG})^{C_t(\text{TG})}$, where $E(\text{UB})$ is the efficiency of the reaction for the universal bacterial primer set; $E(\text{TG})$ is the efficiency of the reaction for the target gene; and C_t is the average C_t value for each sample. Abundance of total fungi, protozoa and bacteria are expressed relative to day 0 of the control period (after feeding the low-fibre, high-PUFA diet for 20 d) and relative to total bacteria, which may account for the differences in DNA integrity and possible PCR inhibitors.

Statistical analysis

Data were analysed using the MIXED procedure of SAS with repeated measures (version 9.3; SAS Institute, Inc.), as described previously[3]. Briefly, the model included sequence and period as random effects, and treatment, time and their interaction as fixed effects. Time was used as the repeated variable and cow by treatment as the subject. The Kenward–Rogers method was used for the adjustment of the denominator df. Furthermore, first-order antedependence covariance structure (allowing unequal spacing) was used in the model. Individual pre-planned contrasts tested control *v.* induction and control *v.* recovery at each time point. Significance and tendencies of main effects and pre-planned contrasts were set at $P < 0.05$ and $P < 0.10$, respectively, and interactions at $P < 0.10$ and $P < 0.15$, respectively. Data were log-transformed (required for all microbial populations), and back-transformed least-squares means and the largest standard error of the means are reported. Second, a random regression model was used to evaluate the associations between rumen microbes and milk fat concentration and milk FA profile, between milk fat concentration and milk FA profile, and between individual groups of rumen microbes from observations on days 0, 5, 9, 13 and 21 using JMP Pro (version 10.0.0; SAS Institute, Inc.). The full model included cow and period as random effects and each microbial taxa as linear and quadratic effects. The quadratic effect was removed from the model when $P > 0.05$ only if the model fit improved based on the Akaike information criterion. Associations with a coefficient of determination > 0.40 are discussed.

Results

Milk production and milk FA profile have been reported previously[3]. Briefly, milk yield was not affected by treatment,

but milk fat yield progressively decreased during the induction period and was 23% lower than the control levels by 7 d and was 34% lower from day 9 to 21. Milk fat yield progressively increased when dairy cows were fed the recovery diet and were not different from that of the control group on day 15. A biphasic response was observed for milk fat *trans* isomers, where the concentrations of *trans*-11 18 : 1 and *cis*-9, *trans*-11-CLA were elevated initially and those of *trans*-10 18 : 1 and *trans*-10, *cis*-12-CLA increased progressively during the induction period. Similarly, a biphasic response was observed during the recovery from MFD, with the concentrations of *trans*-10 18 : 1 and *trans*-10, *cis*-12-CLA rapidly decreasing initially and those of *trans*-11 18 : 1 and *cis*-9, *trans*-11-CLA increasing slightly above the control levels during the second phase of recovery. Treatment × time interactions were detected for all microbial taxa quantified ($P < 0.05$), except for *P. ruminicola* and *B. hungatei*.

Fungi, ciliate protozoa and total bacteria

Total abundance of fungi, ciliate protozoa and bacteria were first determined to observe major shifts in the concentration of rumen microbes (Fig. 1). Fungal abundance decreased by 93% on day 4 of induction, when it reached a nadir ($P < 0.01$; Fig. 1(a)). Conversely, during the recovery period, the abundance of fungi increased progressively, was not different from the control levels on day 8, and reached a plateau on day 12. Similarly, the induction diet progressively decreased the relative abundance of ciliate protozoa, which tended to be lower than the control levels on day 4 ($P = 0.09$; Fig. 1(b)) and reached a nadir on day 8 at a 92% reduction compared with the control diet ($P < 0.01$). Similar trends were observed for fungi and ciliate protozoa relative to total bacteria (see online Supplementary Fig. S1)

There was no overall effect of treatment on the relative abundance of total bacteria, which remained relatively constant during the induction and recovery periods (Fig. 1(c)). However, during the induction period, the abundance of total bacteria tended to be lower than in the control group on day 0 and was higher than in the control group on day 20 ($P = 0.05$ and $P < 0.05$, respectively). During the recovery period, the abundance of total bacteria was higher than that in the control group on days 12 and 20 (1·6-fold increase; $P < 0.05$).

Amylolytic bacteria

The induction diet increased starch concentration, providing more substrate for amylolytic bacteria. During the induction period, the relative abundance of *S. bovis* was 5-fold higher than the control levels on day 4, and remained 3·5-fold higher than the control levels throughout day 20 ($P < 0.01$; Fig. 2(a)) . During the recovery period, the abundance of *S. bovis* was higher than the control levels on day 0 ($P < 0.05$), but was not different from the control levels from day 4 to 20. In contrast, the abundance of *P. bryantii* was not different from the control levels from day 0 to 12 of
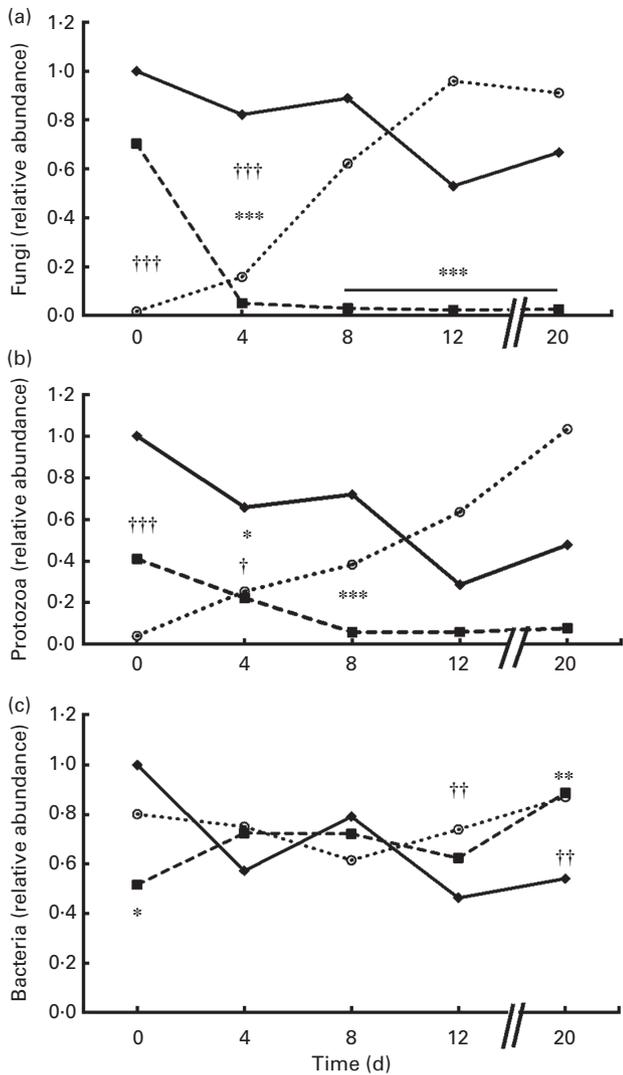
Fig. 1. Time course of the total abundance of (a) fungi, (b) ciliate protozoa and (c) bacteria during the induction of and recovery from milk fat depression. Data are plotted relative to day 0 of the control period. Dairy cows were fed a high-fibre, low-PUFA diet (control; ◆), a low-fibre, high-PUFA diet resulting in milk fat depression (induction; ■), or a high-fibre, low-PUFA diet following induction that resolved milk fat depression (recovery; ○). Values are least-squares means, with their largest standard errors of the mean ((a) sem 0·64, (b) sem 0·91 and (c) sem 0·23). Mean value for the induction group was significantly different from that of the control group: * $P<0·10$, ** $P<0·05$, *** $P<0·01$ (pre-planned contrasts). Mean value for the recovery group was significantly different from that of the control group: † $P<0·10$, †† $P<0·05$, ††† $P<0·01$ (pre-planned contrasts). For fungi and ciliate protozoa, there was a significant main effect of treatment ($P<0·001$ both), but no effect for time ($P=0·45$ and $P=0·54$, respectively), and the treatment × time effect was significant ($P<0·001$ both). For bacteria, there was a significant treatment × time effect ($P=0·02$), but no effects for treatment ($P=0·21$) and time ($P=0·38$).

induction, but was 56 % lower than the control levels on day 20 ($P=0·03$; Fig. 2(b)). During the recovery period, the abundance of *P. bryantii* tended to be lower than the control levels on days 0 and 8 ($P<0·10$) and was 6-fold lower than the control levels on day 4 ($P<0·01$), but was not different on days 12 and 20.

Lactic acid-utilising bacteria

Increasing diet fermentability by the addition of dietary starch results in ruminal lactate production, providing substrate for lactate-utilising bacteria. During the induction period, the abundance of *M. elsdenii* and *S. ruminantium* increased progressively and was higher than in control from day 4 to 12 ($P<0·05$; Fig. 3), but declined thereafter and was not different from control on day 20. The relative abundance of *M. elsdenii* peaked on day 12 of induction (16-fold increase), whereas that of *S. ruminantium* reached near the maximum level on day 8 (1·7-fold increase *v.* control). When dairy cows were switched to the recovery diet, the abundance of *M. elsdenii* declined and was not different from the control levels from day 4 to 20. The abundance of *S. ruminantium* did not differ from that of the control group at any time point during the recovery from MFD.
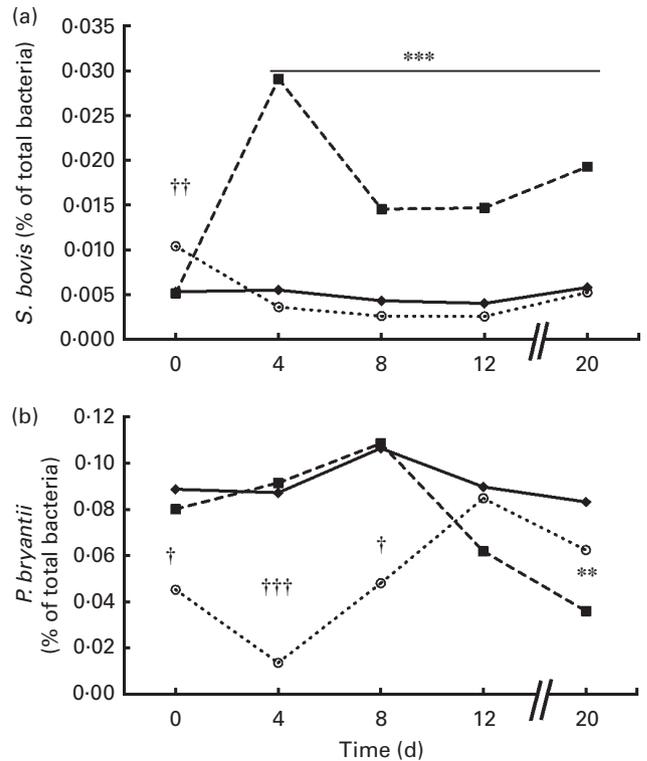
Fig. 2. Time course of the relative abundance of amylolytic bacteria ((a) *Streptococcus bovis* and (b) *Prevotella bryantii*) in the rumen digesta of dairy cows fed a high-fibre, low-PUFA diet (control; ◆), a low-fibre, high-PUFA diet resulting in milk fat depression (induction; ■), or a high-fibre, low-PUFA diet following induction that resolved milk fat depression (recovery; ○). Abundance of the bacterial species is expressed as a percentage of total bacterial 16S ribosomal DNA. Values are least-squares means, with their largest standard errors of the mean ((a) sem 0·017 and (b) sem 0·053). Mean value for the induction group was significantly different from that of the control group: ** $P<0·05$ and *** $P<0·01$ (pre-planned contrasts). Mean value for the recovery group was significantly different from that of the control group: † $P<0·10$, †† $P<0·05$, ††† $P<0·01$ (pre-planned contrasts). For *S. bovis*, there were significant main effects for treatment ($P<0·001$) and time ($P=0·04$), and the treatment × time effect was significant ($P<0·001$). For *P. bryantii*, there was a significant main effect of treatment ($P<0·01$), but no effect for time ($P=0·18$). There was a significant treatment × time effect ($P<0·01$).
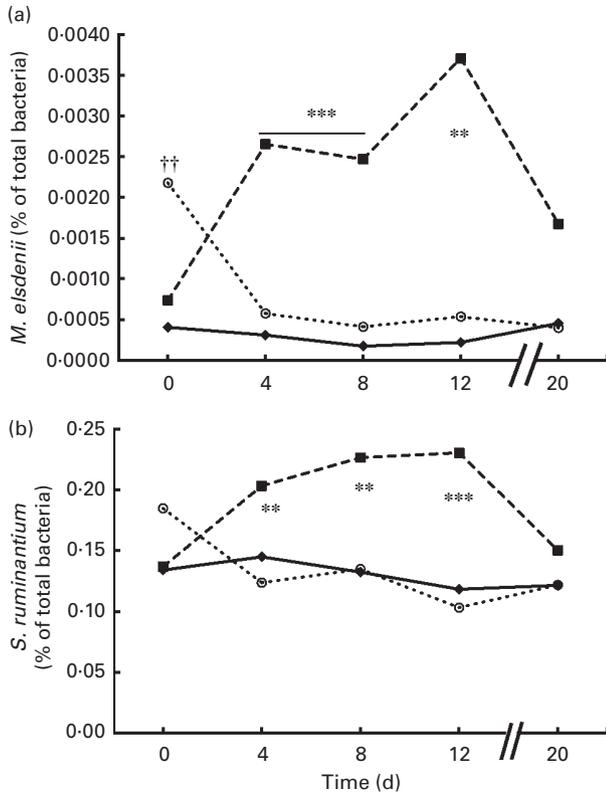
Fig. 3. Time course of the relative abundance of lactate-utilising bacteria ((a) *Megasphaera elsdenii* and (b) *Selenomonas ruminantium*) in the rumen digesta of dairy cows fed a high-fibre, low-PUFA diet (control; ◆–), a low-fibre, high-PUFA diet resulting in milk fat depression (induction; ·-■-·), or a high-fibre, low-PUFA diet following induction that resolved milk fat depression (recovery; ··⊙··). Abundance of the bacterial species is expressed as a percentage of total bacterial 16S rDNA. Values are least-squares means, with their largest standard errors of the mean ((a) SEM 0·0050 and (b) SEM 0·043). Mean value for the induction group was significantly different from that of the control group: ** $P < 0.05$, *** $P < 0.01$ (pre-planned contrasts). †† Mean value for the recovery group was significantly different from that of the control group ($P < 0.05$; pre-planned contrasts). For *M. elsdenii* and *S. ruminantium*, there was a significant main effect of treatment ($P = 0.02$ and $P < 0.01$, respectively), but no effect for time ($P = 0.59$ and $P = 0.34$, respectively). There was a significant treatment × time effect ($P = 0.01$ and $P < 0.01$, respectively).

Fibrolytic bacteria

The induction diet decreased dietary fibre content, but increased the concentrations of unsaturated FA, which are especially toxic to many fibrolytic bacteria. The abundance of *R. albus* did not differ. The abundance of *R. albus* was not different between treatments on day 0 or from day 8 to 20; however, it tended to be lower on day 4 of induction and to be higher on day 4 of recovery relative to the control period ($P = 0.07$ and $P = 0.08$, respectively; Fig. 4(a)). The abundance of *F. succinogenes* decreased by 98 % relative to that of the control group on day 4 of induction, and this level was maintained throughout day 20 ($P < 0.01$; Fig. 4(b)). Conversely, during the recovery period, the abundance of *F. succinogenes* was 99 % lower than that of the control group on day 0 ($P < 0.01$), increased progressively, did not differ from the control levels on day 4, and was fully

recovered by day 12. There was no treatment × time interaction for *P. ruminicola*; however, there was an effect of treatment ($P < 0.01$; see online Supplementary Table S2). The abundance of *P. ruminicola* was 18 % lower than that of the control group during the induction period ($P = 0.02$), and tended to be 20 % higher than that of the control group
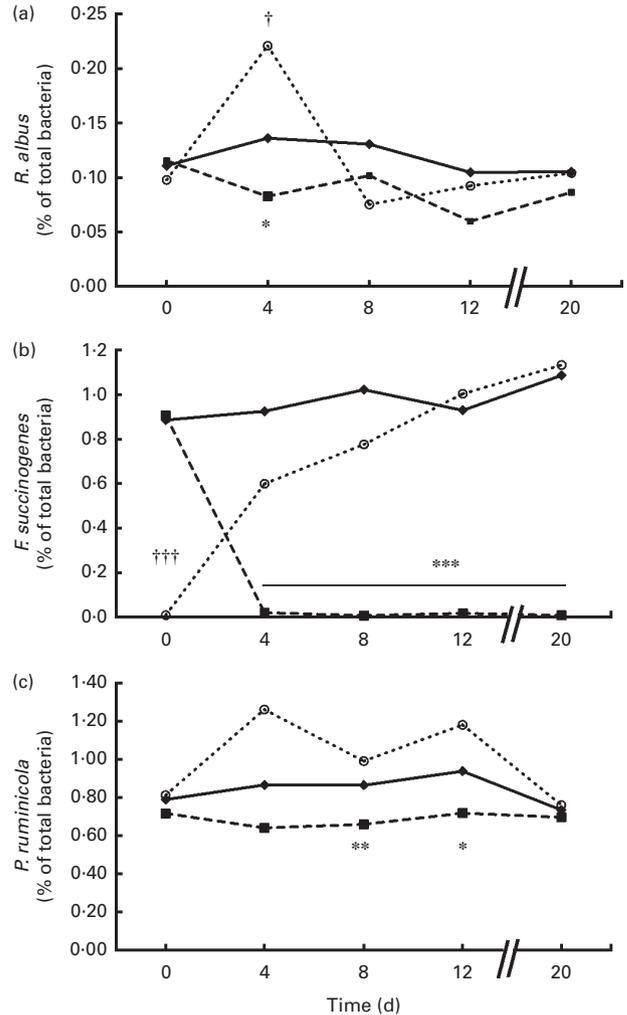
Fig. 4. Time course of the relative abundance of fibrolytic bacteria ((a) *Ruminococcus albus*, (b) *Fibrobacter succinogenes* and (c) *Prevotella ruminicola*) in the rumen digesta of dairy cows fed a high-fibre, low-PUFA diet (control; ◆–), a low-fibre, high-PUFA diet resulting in milk fat depression (induction; ·-■-·), or a high-fibre, low-PUFA diet following induction that resolved milk fat depression (recovery; ··⊙··). Abundance of the bacterial species is expressed as a percentage of total bacterial 16S rDNA. Values are least-squares means, with their largest standard errors of the mean ((a) SEM 0·049, (b) SEM 0·359 and (c) SEM 0·247). Mean value for the induction group was significantly different from that of the control group: * $P < 0.10$, ** $P < 0.05$, *** $P < 0.01$ (pre-planned contrasts). Mean value for the recovery group was significantly different from that of the control group: † $P < 0.10$, ††† $P < 0.01$ (pre-planned contrasts). For *R. albus*, there was a significant main effect of time ($P = 0.02$), but no effect for treatment ($P = 0.24$). There was a significant treatment × time effect ($P < 0.01$). For *F. succinogenes*, there was a significant main effect of treatment ($P < 0.001$), but no effect for time ($P = 0.13$). There was a significant treatment × time effect ($P < 0.001$). For *P. ruminicola*, there was a significant main effect of treatment ($P < 0.01$), but no effect for time ($P = 0.10$). There was no significant treatment × time effect ($P = 0.59$).

during the recovery period ($P = 0.06$). Based on the preplanned contrast, the abundance of *P. ruminicola* was found to be lower than that of the control group on day 8 of induction ($P = 0.04$; Fig. 4(c)), and tended to be lower on day 12 ($P = 0.08$). During the recovery period, the abundance of *P. ruminicola* did not differ from that of the control group at any time point.

Trans-11 18 : 1-producing bacteria

The abundance of the *B. fibrisolvens/Pseudobutyrivibrio* group and *B. hungatei* were investigated in the present study because of their role in ruminal BH of *cis*-9, *cis*-12 18 : 2 (linoleic acid; LA) to *cis*-9, *trans*-11-CLA and *trans*-11 18 : 1[12]. The abundance of the *B. fibrisolvens/Pseudobutyrivibrio* group decreased progressively during the induction period and reached a nadir on day 8 when it was 60 % lower than that of the control group ($P < 0.05$; Fig. 5(a)). There was no treatment × time interaction or treatment effect at any time point for the abundance of *B. hungatei* (see online Supplementary Table S2; Fig. 5(b)).

Associations between milk fat concentration, milk fatty acid profile and microbial taxa

Quadratic relationships were observed between milk fat concentration and anaerobic fungi, *F. succinogenes* and the *B. fibrisolvens/Pseudobutyrivibrio* group ($R^2 \geq 0.52$; Table 2), whereas *trans*-10 18 : 1 concentration (% of C18 FA) was quadratically associated with milk fat concentration, ciliate protozoa, anaerobic fungi, *F. succinogenes* and the *B. fibrisolvens/Pseudobutyrivibrio* group ($R^2 \geq 0.45$). There was a modest quadratic relationship between *trans*-11 18 : 1 (% of C18 FA) and milk fat (R^2 0.34), but *trans*-11 18 : 1 was not strongly associated with any microbial taxa ($R^2 < 0.13$; see online Supplementary Table S3). The relationship between microbial populations was also investigated by regression analysis (see online Supplementary Table S3). Briefly, anaerobic fungi were correlated with protozoa (R^2 0.57) and *F. succinogenes* (R^2 0.76). Also, modest correlations were observed between protozoa and *F. succinogenes* and between *S. ruminantium* and the *B. fibrisolvens/Pseudobutyrivibrio* group ($R^2 > 0.33$).

Discussion

Previous investigations have predominantly focused on characterising the gastrointestinal microbiome after adaptation to a diet rather than on temporal events occurring during adaptation[13–15], although Tajima *et al.*[5] reported an experiment with a low-resolution time course (i.e. days 0, 3 and 28) on the changes in select bacterial groups after switching cows from a high-fibre diet to a high-grain diet. The repeated-measures design adopted in the present experiment accounted for the variation in cow and period, and strengthens the ability to investigate the time course of adaptation. To understand the variation due to cow and period, we ran the model using the sum-of-squares estimation (type 3) without a repeated statement. On average, period was 4.8 % of the sum of squares
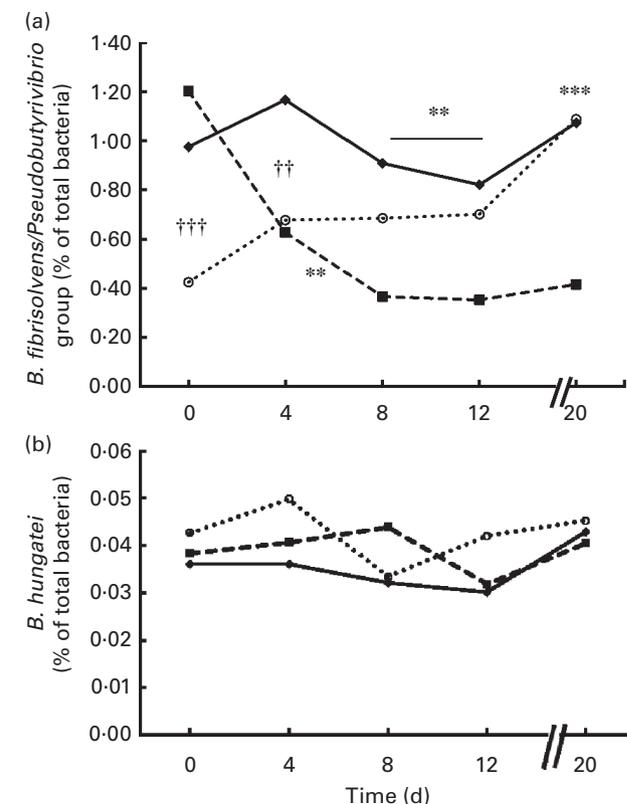
Fig. 5. Time course of the relative abundance of *trans*-11 18 : 1-producing bacteria ((a) *Butyrivibrio fibrisolvens/Pseudobutyrivibrio* group and (b) *Butyrivibrio hungatei*) in the rumen digesta of dairy cows fed a high-fibre, low-PUFA diet (control; ◆—), a low-fibre, high-PUFA diet resulting in milk fat depression (induction; ■--), or a high-fibre, low-PUFA diet following induction that resolved milk fat depression (recovery; ⊙··). Abundance of the bacterial species is expressed as a percentage of total bacterial 16S rDNA. Values are least-squares means, with their largest standard errors of the mean ((a) SEM 0.273 and (b) SEM 0.017). Mean value for the induction group was significantly different from that of the control group: ** $P < 0.05$, *** $P < 0.01$ (pre-planned contrasts). Mean value for the recovery group was significantly different from that of the control group: †† $P < 0.05$, ††† $P < 0.01$ (pre-planned contrasts). For the *B. fibrisolvens/Pseudobutyrivibrio* group, there was a significant effect of treatment ($P < 0.01$), but no effect for time ($P = 0.15$). There was a significant treatment × time effect ($P < 0.001$). For *B. hungatei*, there was no significant effect for treatment ($P = 0.52$) or time ($P = 0.33$), and no significant treatment × time effect ($P = 0.67$).

and cow was 16.5 % of the sum of squares. Cow variability was 0.8–38 % of the total sum of squares depending on the microbial population. Previous work reporting milk fat and milk FA profile every other day when cows were switched to a high-fat diet[2] provided insight into temporal changes in rumen metabolism and thus the expected changes in ruminal microbial populations. Samples were collected to represent different periods of the *trans* isomer response including the peak concentration of *trans*-11 18 : 1 (day 4), with *trans*-10 18 : 1 becoming the predominant *trans* isomer (days 8 and 12), and established MFD (day 20). An additional sample was collected on day 16, but was not analysed because preliminary analysis of one cow indicated insignificant difference between days 12, 16 and 20. Thus, the measurements of time course in the present study help in the mechanistic interpretation of microbial adaptation associated with MFD

Table 2. Associations between milk fat and *trans*-10 18 : 1 concentrations and selected rumen microbes

Response/predictor†	R^2	Adjusted R^2	RMSE	Estimates			P*		
				Int	L	Q	Int	L	Q
Milk fat (%)									
Trans-10 18 : 1 (% of C18 FA)	0·71	0·71	0·42	4·04	−0·187	0·00931	<0·001	<0·001	<0·001
Trans-11 18 : 1 (% of C18 FA)	0·34	0·33	0·62	2·59	0·392	−0·144	<0·001	<0·001	<0·001
Ciliate protozoa	0·54	0·53	0·52	2·85	0·214	−0·0209	<0·001	<0·001	<0·001
Anaerobic fungi	0·70	0·69	0·43	2·91	4·08	−10·7	<0·001	<0·001	<0·001
Streptococcus bovis	0·20	0·18	0·69	3·62	−18·0	107	<0·001	<0·01	0·01
Prevotella bryantii	0·06	0·05	0·72	3·51	−0·931	–	<0·001	0·17	–
Megasphaera elsdenii	0·07	0·05	0·69	3·49	−9·10	6·80	<0·001	<0·01	<0·01
Selenomonas ruminantium	0·25	0·25	0·65	3·91	−3·13	–	<0·001	<0·001	–
Ruminococcus albus	0·16	0·14	0·69	3·11	3·52	−22·0	<0·001	<0·01	<0·001
Fibrobacter succinogenes	0·57	0·56	0·51	2·97	0·894	−0·458	<0·001	<0·001	<0·001
Prevotella ruminicola	0·11	0·09	0·72	3·09	0·426	−0·322	<0·001	0·10	0·04
B. fibrisolvens/Pseudobutyrivibrio group	0·52	0·51	0·53	2·53	1·24	−1·09	<0·001	<0·001	<0·001
Butyrivibrio hungatei	0·17	0·15	0·69	3·57	−0·957	−130	<0·001	0·80	0·03
***Trans*-10 18 : 1 (% of C18 FA)**									
Ciliate protozoa	0·56	0·55	3·12	7·88	−1·46	0·182	<0·001	<0·001	<0·001
Anaerobic fungi	0·63	0·63	2·93	7·36	−24·2	64·8	<0·001	<0·001	<0·001
Streptococcus bovis	0·30	0·28	4·06	2·76	144	−673	0·05	<0·001	<0·01
Prevotella bryantii	0·05	0·04	4·53	3·89	5·84	–	<0·01	0·18	–
Megasphaera elsdenii	0·38	0·36	3·70	3·65	77·2	−54·4	0·02	<0·001	<0·001
Selenomonas ruminantium	0·33	0·32	3·89	0·974	22·2	–	0·25	<0·001	–
Ruminococcus albus	0·21	0·20	4·16	7·67	−33·0	144	<0·001	<0·001	<0·001
Fibrobacter succinogenes	0·59	0·58	3·07	7·40	−5·88	2·83	<0·001	<0·001	<0·001
Prevotella ruminicola	0·07	0·06	4·53	5·39	−1·01	–	<0·001	0·37	–
B. fibrisolvens/Pseudobutyrivibrio group	0·45	0·44	3·45	8·78	−6·73	8·08	<0·001	<0·001	<0·001
Butyrivibrio hungatei	0·17	0·15	4·30	6·47	−66·1	1421	<0·001	<0·001	<0·001

RMSE, root mean squared error; Int, intercept; L, linear coefficient; Q, quadratic coefficient; FA, fatty acid.
* Significance of the intercept, linear and quadratic terms. The quadratic effect was removed from the model when $P > 0.05$ only if fit improved based on the Akaike information criterion.
† Predictors are expressed as a percentage of total bacteria.

and modification of BH pathways. Importantly, this time course allows the separation of primary responses from secondary adaptations.

Dietary components have an impact on the ruminal environment and predominant microbial populations, which, in turn, affect the rate and pathways of BH[12,16]. The induction diet was designed to cause MFD using dietary conditions that commonly occur on farms. The experimental design was successful in the induction of and recovery from MFD within the experimental periods. Although not measured in the present study, ruminal pH was expected to have been lower during the induction period due to increased dietary starch and decreased forage NDF. Decreased ruminal pH results in changes in the microbial population (specifically cellulolytic bacteria) and BH pathways, resulting in the increased formation of *trans*-10 18 : 1 and *trans*-10, *trans*-12-CLA[17]. The induction diet also had a high concentration of rapidly available PUFA, specifically LA (3·4 % DM), which is known to have a negative impact on bacterial membrane integrity and the growth of numerous species of ruminal bacteria[18,19]. Increasing PUFA also promotes a shift in microbial communities and BH pathways towards those that cause MFD[4,12]. Given that the bacterial species assayed in the present study represent a small proportion of the total community, cause-and-effect relationships cannot be drawn from the present study. However, we propose that the timing of changes in all the identified taxa, including protozoa and fungi, provide an indication of the timing of the global

adaptation of the rumen microbiome previously reported to occur during MFD[4].

Ciliate protozoa are the largest and most important group of protozoa in the rumen[20], and anaerobic fungi (order Neocallimastigales) encompasses several families and species with diverse activities[21]. Both ciliate protozoa and fungi were acutely responsive to the induction and recovery diets, and demonstrated the rapid adaptation of the rumen to the change in the diet. The regression analysis revealed that milk fat concentration quadratically increased with increasing number of protozoa, peaked when protozoa were approximately 6 % of total bacteria, and then decreased with a greater number of protozoa; whereas milk fat *trans*-10 18 : 1 concentration quadratically decreased with increasing number of protozoa, and reached a nadir when the number of protozoa was about 4 % of total bacteria (Table 2). Rumen ciliate protozoa are known to contain high concentrations of *trans*-11 18 : 1 and CLA[22,23]; however, they do not directly contribute to the BH of LA[23] and were not correlated with the concentration of *trans*-11 18 : 1 in milk fat in the present study (R^2 0·08; $P = 0.09$; see online Supplementary Table S3).

It is unclear whether the rapid reduction in the abundance of ciliate protozoa and fungi during the induction of MFD in the present study is due to the high-PUFA diet, high diet fermentability, or their interaction. The reported effects of high-grain diets and low ruminal pH on rumen protozoa are contradictory. Low ruminal pH under high-grain diet conditions has been previously shown to have a negative

impact on the abundance of rumen protozoa *in vitro* [24] and *in vivo* [25,26]. In contrast, other studies have observed positive effects of high-grain diets on the abundance of protozoa [27,28]. Dietary PUFA have been reported to be toxic to protozoa populations, although the degree of inhibition is greater for linseed than for other oils such as soyabean and rapeseed [29]. In agreement with our observations under the MFD induction diet condition, feeding a ration containing sunflower oil (6% DM) decreased ciliate protozoa concentrations in ruminal fluid by 80% within 6 d of feeding [30].

Very few data exist on the effects of diet on ruminal fungi and their role in ruminal BH, although some species (e.g. *Neocallimastix frontalis*) are known to metabolise LA to *trans*-9, *trans*-11-CLA, whereas the growth of others (e.g. *Piromyces communis*) is inhibited by LA [18]. Using automated ribosomal intergenic spacer analysis, Boots *et al.* [31] recently investigated the effects of increasing dietary concentrates with or without the addition of soyabean oil (6% DM), and reported a more pronounced reduction in anaerobic fungal diversity in the rumen when oil was increased compared with when concentrate was increased.

The abundance of *F. succinogenes*, *P. ruminicola*, *M. elsdenii*, *S. ruminantium* and *S. bovis* in the control group was similar to that reported previously by Stevenson & Weimer [6], whereas the abundance of *R. albus* was higher and that of *P. bryantii* was lower. Interestingly, although *M. elsdenii* is considered a major lactic acid user in the rumen [32], its relative abundance was much lower than that of *S. ruminantium* in the present experiment, similar to that reported previously [6]. Although not measured, lactic acid concentration in the rumen was expected to have been increased under the MFD induction diet condition. Compared with high-forage diets, feeding high-grain diets increased the formation of lactic acid by ruminal bacteria such as *S. bovis* [33,34], which drastically increased during the induction period. In agreement with our observations, the abundance of *M. elsdenii* was also elevated in milk fat-depressed cows receiving a low-forage diet containing flaked 'corn' [35], in cows fed high-grain diets [26], and in two cows with low milk fat fed a monensin-supplemented high-'corn' silage diet [36]. The more pronounced increased in the abundance of *M. elsdenii* in the present experiment could be related to its lower sensitivity to the toxic effects of PUFA [18]. Interestingly, both species decreased to near control values by day 20 of induction, suggesting a possible secondary adaptation to the diet, which is supported by a decrease in the abundance of *S. bovis* after day 4, or a long-term detrimental effect of the diet on the growth of the lactate users. In addition, we observed that *trans*-10 18:1 in milk fat was quadratically related to *M. elsdenii* (increased with increasing abundance of *M. elsdenii* and peaked when *M. elsdenii* was approximately 0·7% of total bacteria), whereas it increased linearly with increasing abundance of *S. ruminantium*. The YJ4 strain of *M. elsdenii* has been reported to produce *trans*-10, *trans*-12-CLA from linoleic acid [37], although others have reported that other strains do not possess this ability [38]. However, *Propionibacterium acnes* has been suggested to be a more likely candidate for ruminal synthesis of *trans*-10, *cis*-12-CLA [12], and other unculturable species probably are major contributors.

When transitioning cows from a high-hay to a high-grain diet, Tajima *et al.* [5] observed a sharp initial increase in the abundance of the amylolytic bacteria *S. bovis* and *P. bryantii* on day 3 and a large reduction in the number of both bacterial species by day 28. In contrast, in the present experiment, *S. bovis* and *P. bryantii* responded differently during the induction period. While the two bacterial species degrade starch avidly [39,40], *P. bryantii* also exhibits xylanase and pectinase activities [41] and appeared to be part of the second phase of rumen adaptation, whereas *S. bovis* appeared to be part of the first phase. This difference in response may be explained by the higher sensitivity of *P. bryantii* to PUFA compared with *S. bovis* [18]. Temporally, the peak abundance of *S. bovis* coincided with peak milk concentration of vaccenic acid, and the decline in the abundance of *S. bovis* after day 4 coincided with the shift to the alternative BH pathway [3]. However, *S. bovis* was poorly correlated ($R^2 < 0.30$) with milk *trans*-11 18:1 concentration, but was correlated with milk fat concentration (quadratic and increased with the abundance of *S. bovis* above 0·08% of total bacteria) and *trans*-10 18:1 concentration (quadratic and decreased with the abundance of *S. bovis* above 0·1% of total bacteria). Although *S. bovis* is not known to be important in the ruminal formation of *trans*-11 18:1 or CLA [18], it is known to biohydrogenate MUFA and PUFA [42]. Interestingly, the decline in the abundance of *P. bryantii* during the induction period coincided with the alternative BH pathway that became the predominant pathway, and its increase during the induction period coincided with the decline in the alternative pathway and re-establishment of the normal pathway. However, it was poorly correlated with the concentrations of milk *trans*-10 18:1 and milk fat.

The fibrolytic species of bacteria selected in the present experiment also differed in their response to dietary changes possibly due to differences in sensitivity to ruminal pH and PUFA. In agreement with the present results, Tajima *et al.* [5] reported a dramatic reduction in the abundance of *F. succinogenes* on day 3 of feeding a high-grain diet, which was further reduced by day 28. The rapid decline in the abundance of *F. succinogenes* coincided with the increase in the concentration of milk fat *trans*-11 18:1, but was poorly correlated to this FA, and further changes were not observed during the shift to the alternative pathway. The increase in the abundance of *F. succinogenes* during the recovery from MFD temporally matched the decline in the alternative BH pathway. In contrast, *P ruminicola* seemed to be less responsive to dietary changes in the present experiment as it was only mildly reduced during the induction period and was poorly associated with the concentrations of milk fat and *trans*-10 18:1. However, *P. ruminicola* differs from other more specialised cellulolytic bacteria as it has the ability to ferment peptides and amino acids. Interestingly, *R. albus* was only affected on day 4 of the induction and recovery periods, and may represent a transitory role in rumen adaptation.

Ruminal bacteria are generally considered to be the main group accounting for ruminal BH of unsaturated FA [12]. Both the *B. fibrisolvens/Pseudobutyrivibrio* group and *B. hungatei*

produce *trans*-11 18:1[43,44], although in the present experiment, both were poorly associated with *trans*-11 18:1 (*B. fibrisolvens/Pseudobutyrivibrio* group R^2 0·13 and *B. hungatei* R^2 0·03). Despite their commonalities with regard to BH products and their phylogenetic proximity, *B. hungatei* is known to have higher butyrate kinase activity and to be more sensitive to growth inhibition by LA compared with the *B. fibrisolvens/Pseudobutyrivibrio* group[43]. The inclusion of 2% sunflower oil in the diet of dairy ewes had no effect on the relative abundance of *trans*-11 18:1-producing *Butyrivibrio*. In contrast, the abundance of *B. fibrisolvens/Pseudobutyrivibrio* group progressively decreased during the induction of MFD in the present experiment, whereas no changes were observed in the abundance of *B. hungatei*. It is possible that the negative effects of the MFD induction diet on the abundance of *B. fibrisolvens/Pseudobutyrivibrio* group were related to the reduction in dietary fibre content, as *B. fibrisolvens*-related bacteria are known to be involved in fibre digestion[45]. The gradual decline in the abundance of the *B. fibrisolvens/Pseudobutyrivibrio* group during the induction period temporally matched the shift to the alternative BH pathway, although the gradual recovery lagged behind the recovery of the normal pathway.

Conclusions

Changes in dietary fibre and unsaturated FA concentrations resulting in the induction of and recovery from milk fat depression also cause a rapid alteration of ruminal microbial populations. Only a small proportion of total bacteria were evaluated; however, the time course of rumen taxa modifications and their correlations with milk fat and milk *trans*-FA concentrations are expected to reflect the time course of global microbial shifts. Changes in the rumen microbiome may be the functional and rate-limiting step in the induction of and recovery from diet-induced milk fat depression; however, further investigation is needed to elucidate the functional role of specific bacterial populations in the process of modification of dietary lipids.

Supplementary material

To view supplementary material[46–49] for this article, please visit http://dx.doi.org/10.1017/S0007114515001865

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