1 Substituting imported soybean meal with locally produced novel yeast protein in concentrates

2 for Norwegian Red dairy cows: Implications for microbiota and fatty acid composition

3 Eirin Stork, Dag Ekeberg, Hanne M. Devle, Özgün C. O. Umu, Davide Porcellato, Martine

4 A. Olsen, Stine G. Vhile, Alemayehu Kidane, Tove Devold and Siv B. Skeie

5 SUPPLEMANTARY FILE

6 **Supplementary materials and methods**

7 **Experimental design**

8 The feeding experiment was performed at the Livestock Production Research Centre at the

9 Norwegian University of Life Sciences (NMBU, Ås, Norway), and approved by the national

10 animal research authority of the Norwegian Food Safety Authority (FOTS ID: 18038).

11 A detailed description of the feeding experiment is previously described in Olsen *et al*.

12 (2021) and Kidane *et al*. (2022). In short, a total of 48 NR dairy cows in their early to mid-

13 lactation (103 ± 33.5 days in milk (DIM)) were allocated into three different feeding

14 treatments (16 animals in each group) based on DIM, parity, milk yield at start of the

15 experiment and milk protein genetic variants. The feeding treatments consisted of the same

16 basal diet of grass silage (65% on average of total dry matter intake (DMI)), but different

17 concentrate feeds (35% on average of total DMI). The concentrate feeds were all based on

18 barley as the main ingredient ($48.9 - 55.4\%$), but 7% of the barley in the barley-feed (BAR,

19 used as negative control) was replaced by either soybean meal in the conventional soybean

20 meal-based feed (SBM, used as control) or yeast microbial protein in the yeast-based feed

21 (YEA). The concentrates were produced to be approximately isoenergetic, with SBM and

22 YEA containing the same amount of protein. The yeast *Cyberlindnera jadinii* used in this

23 experiment was produced by Danstar Ferment (Fredericia, Denmark) using sugar cane

24 molasses as growth medium. The fat supplement used in the concentrate feeds was Akofeed

25   Lac 45; calcium soaps of approximately 1% C14:0, 47% C16:0, 6% C18:0, 37% C18:1 and

26   9% C18:2, produced by AAK (Malmö, Sweden). Akofeed constituted 3.04% of the YEA,

27   3.29% of the BAR and 3.38% of the SBM (Kidane *et al*. 2022). The amount varied slightly

28   because the concentrates were made to be isoenergetic.

29   The experiment lasted for a total of 10 weeks with the first 2 weeks considered as an

30   adaptation period where all cows were fed the same control diet (grass silage and SBM),

31   before the cows were allocated to experimental diets for a period of 8 weeks. The cows had

32   free access to grass silage throughout the experiment from automatic feeders, and the feed

33   intake was recorded for each individual cow. The amount of concentrate feed for each

34   individual cow was calculated using the NorFor feeding system (Volden, 2011) and fed in

35   several portions each day from an automatic feeding system. To account for the increasing

36   lactation stage and decreasing yield, the amount of concentrate feeds given to the cows was

37   adjusted twice over the experimental period (first reduced 15% on day 28, and then reduced

38   again 10% on day 50, relative to the adaptation period) for all groups. The feeding

39   experiment as such with ingredients and chemical composition of grass silage and the three

40   concentrate feeds has been published by Kidane *et al*. (2022) and more details can be found

41   there.

42   **Feed sampling**

43   Samples from each of the concentrate feeds (approx. 400 g), as well as the grass silage

44   (approx. 500 g), were taken once every week and stored at -20°C until further processing for

45   analysis of the fatty acid (FA) composition by GC-MS. The samples were pooled and dried at

46   45°C for 48 h and milled using a cutting mill (Retsch SM 200, Retsch GmbH, Germany) to a

47   sieve size of 1.0 mm for FA analysis. The feed samples were further stored in darkness at 4°C

48   until analysis. Three replicates of 1.00 g of each feed (SBM, BAR, YEA and grass silage)

49   were weighed out and prepared for GC-MS analysis.

Milk sampling

The cows were milked using an automatic milking system (De Laval, Lund, Sweden) with

access every 6th hour, with a maximum of four milking's per day. The automatic milking

system is equipped with a sampler that draws a representative sample throughout milking.

Separate milk samples were drawn from every milking from Monday morning to Wednesday

morning in experimental weeks 2, 4, 6, 7 and 10. For each sampling period (week) the

samples were collected, pooled and mixed for each individual cow. 1.00 g of the samples

from each cow per week was weighed out into glass tubes and stored in darkness at -20°C

until further analysis of FA composition. Milk samples from weeks 2, 6 and 10 were analysed

for FA composition. All milk samples were prepared and analysed in random order to prevent

systematic errors.

Extraction, derivatization, and GCMS analysis of lipids

Chemicals and standards

All solvents used were of chromanorm quality from VWR Chemicals (Radner,

Pennsylvania), but the 14% solution of boron-trifluoride in methanol used for the methylation

of lipids, was supplied by Sigma Aldrich (Switzerland). Supelco 37-component FAME mix

(Supelco, Schnelldorf, Germany) was used for the identification of fatty acid methyl esters

(FAMEs) in the milk samples. Further identification of FAs not included in this 37-

component FAME mix was performed by the following individual FAME standards: methyl

heptanoate, methyl nonanoate, methyl 11-methyldodecanoate, methyl 12-methyltridecanoate,

methyl 13-methyltetradecanoate, methyl 14-methylpentadecanoate, *trans*-9-hexadecenoic

acid methyl ester, *cis*-7-hexadecenoic acid methyl ester, methyl 14-methylhexadecanoate,

methyl 3,7,11,15-tetramethylhexadecanoate, *cis*-9-heptadecenoic acid methyl ester, 16-

methylheptadecanoic acid methyl ester, *trans*-11-octadecenoic acid methyl ester, *cis*-11-

octadecenoic acid methyl ester, cis-13-octadecenoic acid methyl ester, *cis*-10-nonadecenoic

75   acid methyl ester, *cis*-9,*trans*-11-octadecadienoic acid methyl ester, all *cis*-8,11,14,17-

76   eicosatetraenoic acid methyl ester and all *cis*-7,10,13,16,19-docosapentaenoic acid methyl

77   ester (all from Larodan AB, Malmö, Sweden).

78   The triacylglyceride triundecanoin (C11:0 TAG) (Larodan AB, Malmö, Sweden) was chosen

79   as internal standard (IS) for the quantification of FAMEs in milk. The stock solution was

80   prepared by dissolving 100 mg of IS in 10 mL chloroform to a total concentration of 10

81   mg/mL. The solution was transferred to GC-vials and stored in darkness at -20°C until use.

82   *Lipid extraction and derivatization to FAMEs*

83   A modified version of the Folch extraction method as described by Devle *et al*. (2014),

84   explained by the following procedure, was used to extract the lipids from the samples. Prior

85   to extraction, 150 µL of the IS stock solution was added to 1.00 g of the thawed milk sample

86   and mixed using a vortex mixer. IS was not used for the feed samples. For the extraction of

87   the lipids, 20 mL of chloroform:methanol (2:1, v/v) was added to the sample and shaken

88   horizontally on an orbital shaker at 350 rpm for 20 minutes. 4 mL of 0.9% sodium chloride in

89   milli-Q water was then added to wash out the more polar components in the sample. After

90   mixing, the sample was centrifuged at 670 rcf for 5 minutes, and the resulting upper aqueous

91   phase with the more polar components was discarded. The lipid-containing organic phase left

92   in the tube was evaporated to dryness at 40°C using a gentle stream of nitrogen. Chloroform

93   was then added to dissolve the lipids before the extract was transferred to a screw capped

94   culture tube (Duran GL14), which again was placed in a heating block at 40°C under a gentle

95   stream of nitrogen until complete removal of the solvent. The dried residue was redissolved

96   in 1.0 mL of heptane for further transesterification of the lipids to FAMEs. The

97   transesterification procedure was performed by adding 1 mL of sodium methanolate solution

98   (5 mg/mL) to the culture tube with a screw cap and placing the sample horizontally on an

99   orbital shaker at 390 rpm for 20 minutes. 1 mL of a boron trifluoride solution (14%) was then

100   added to the sample and the screw capped culture tube was placed in a water bath at 80°C for

101   20 minutes. The sample was then left to cool down, before it was centrifuged for 5 minutes at

102   220 rcf. The upper heptane phase was collected in a GC-vial and stored in a freezer at -20°C

103   until GC-MS analysis.

104   ***GC-MS Analysis of FAMEs***

105   The GC used was a TRACE 1310 (Thermo Fisher Scientific, Waltham, MA, USA), equipped

106   with an AI/AS 1310 Series Autosampler (Thermo Fisher Scientific, Waltham, MA, USA),

107   supplied with a 60 m Rtx-2330 capillary column (90% biscyanopropyl and 10%

108   phenylcyanopropyl polysiloxane) with ID 0.25 mm and 0.20 µm film thickness (Restek,

109   Bellefonte, PA, USA). Helium (99.99990%, AGA, Norway) was used as carrier gas and set

110   to a constant flow of 1.0 mL/min. 1.0 µL of the sample was transferred to the injection

111   chamber at 250°C in split mode, with a split ratio of 1:10. The split flow was set to 10.0

112   mL/min and the purge flow set to 5.0 mL/min. The GC oven was temperature programmed,

113   starting at 50°C for 5 min, before the temperature was increased with a rate of 100°C/min to

114   140°C, where it was held for 30 min. The temperature was further increased to 145°C at a

115   rate of 10.0°C/min for 30 min, before it again was increased to 175°C at a rate of 3°C/min for

116   20 min. Finally, the temperature was increased at a rate of 50°C/min to the highest

117   temperature of 260°C and held there for 10 min. The total run time was 108 min. Chromeleon

118   v7.2.9 (Thermo Fisher Scientific, Waltham, MA, USA) was used as software for the analysis.

119   For the identification of FAMEs in the sample, an ISQ QD GC-MS (Thermo Fisher

120   Scientific, Waltham, MA, USA) was used. The MS was a single quadrupole, using an

121   electron ionization energy of 70 eV. The mass range was *m/z* 50 – 600, and full-scan

122   acquisition mode was used with 0.2 sec scan time. The MS transfer line and ion source were

123   both set at 250°C. NIST 17 Mass Spectral Library (Gaithersburg, MD, USA) was used as

124   reference library for the identification of the FAMEs present in the sample, together with the

125 retention times of the standards present in the FAME mix and the individual FAME

126 standards. FAs identified by library search only, are marked with a superscript letter in all

127 tables. The milk FAs were quantified using the amount of internal standard added and single-

128 point calibration together with predetermined relative response factors, and the results are

129 presented as g/100 g of total FAs. The results from the FA analysis of the feeds are presented

130 as relative amounts given in percent of total based on the area of the peaks.

131 **Statistical Analysis**

132 The statistical analysis of the milk data was performed using RStudio (version 1.4.1103,

133 2009-2021, PBC, Boston, MA). Significant effects ($P < 0.05$) of experimental feed and week

134 into the experiment (6 or 10) on the FA composition were analysed using the lmer-function

135 of the lme4-package to fit the data to a linear mixed-effects model. The values from the SBM

136 control group were used as reference. Experimental feed and week into the experiment were

137 regarded as fixed effects, while cow was regarded as a random effect. Repeated

138 measurements were correlated in the model. The measured values in the control period (week

139 2) were considered as covariates. Data values from the FA analysis of one cow in the BAR-

140 group were excluded from the statistical analysis, because the value from the control period

141 was missing due to sample loss.

142 The linear model used is described by this equation:

$$y_{ijk} = \mu + \gamma_i + \tau_j + K_k + E_{ijk}$$

144 Were y_{ijk} is the response variable, in this case, the difference in the content of the FAs or FA

145 groups between the experimental period and the control period, μ is the overall mean, γ_i is

146 the effect of the feed, τ_j is the effect of week, K_k is the effect of cow and E_{ijk} is the error-

147 term.

148   The effects of concentrate feed on sensory data were tested using the logistic procedure in

149   SAS Enterprise Guide 7.1 (SAS, Cary, USA). The model used included the fixed effects of

150   concentrate feed (BAR, SBM or YEA), week (4, 6, 7, 10), parity (primiparous or

151   multiparous), covariate (week 2), and the interaction between concentrate feed and week.

152   **Microbiota Analysis**

153   *DNA extraction and 16S rRNA gene sequencing*

154   The DNA extraction from the rumen samples was performed using QIAamp PowerFecal Pro

155   DNA Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol. The

156   extracted DNA was quantified by using Qubit™ fluorometer and dsDNA BR or HS Assay

157   Kits (Invitrogen, Eugene, OR, USA) and Gel electrophoresis with 1% agarose gel was used

158   to check the quality of the DNA. The DNA samples were sent to Eurofins Genomics

159   (Ebersberg, Germany) or processed at NMBU for library preparation and 16S rRNA gene

160   sequencing for rumen microbiome profiling. The V3-V4 variable region of the bacterial 16S

161   rRNA gene was amplified using Uni340F (CCTACGGGRBGCASCAG) and Uni806R

162   (GGACTACYVGGGTATCTAAT) primers. The PCR amplification and library preparations

163   were similar to Skeie *et al.* (2019) and sequencing was performed on an Illumina MiSeq

164   machine (Illumina, San Diego, CA, USA) with an output of 2 x 300 base-pair paired-end

165   reads. The fastq files have been deposited at the European Nucleotide Archive with accession

166   number PRJEB53992. The analysis of the demultiplexed paired-end raw reads was carried

167   out using DADA2 R package (version 3.11) (Callahan *et al*. 2016). The primers were

168   checked and removed, reads were filtered and trimmed to remove the low-quality tails at the

169   truncation length of 250bp for both forward and reverse reads and using the parameters of

170   maxEE of 2 and truncQ of 2. The core DADA2 algorithm was used for dereplication and

171   denoising of the quality-filtered reads (the average number of 25189 ± 11969 reads for the

172   rumen samples, and the paired reads were merged. The chimeric ASVs were identified and

173 removed from the Amplicon Sequence Variant (ASV) table constructed. Ribosomal Database

174 Project (RDP) Naive Bayesian Classifier algorithm (Wang *et al*. 2007) implemented in

175 DADA2 R package was used for taxonomy assignment to the ASVs using default settings

176 and Silva prokaryotic SSU taxonomic training data formatted for DADA2 with species

177 (release-138.1) (https://zenodo.org/record/4587955#.YIqLrLUzZPY) (Quast *et al*. 2013). A

178 Phyloseq object was created from the ASV, taxonomy and metadata tables using phyloseq R

179 package (version 1.40.0) (McMurdie & Holmes, 2013). The features that were assigned to

180 Archaea, Chloroplast and Mitochondria were filtered by taxonomy-based filtering.

181 The filtered ASV tables were preprocessed using the multivariate statistical framework

182 mixMC (Cao *et al.* 2016) in mixOmics R package (version 6.19.4) (Rohart *et al*. 2017). For

183 the pre-processing of the data, an offset of 1 to the whole data matrix was added to deal with

184 the zeros, the low count ASVs were removed across all samples using the cutoff of 0.01

185 percent, and the Centered-Log Ratio (CLR) transformation was applied. Principle Component

186 Analysis (PCA) was performed to identify the variation in the microbiome composition over

187 time and based on the diet groups.

188 The taxa were agglomerated at genus level and lmmsDE function in R package lmms

189 (version 1.3.3) was used to fit linear mixed effect model splines to perform differential

190 abundance analysis. Differential abundance of bacterial groups was analysed over time,

191 between feeding groups and for the interaction of time and group.

Supplementary results

Supplementary table S1. The fatty acid (FA) profile (% of total FAs based on the peak area, given as the average of three replicates ± standard deviation) of grass silage and three concentrates (SBM = barley-based with additional protein from soybean meal, BAR = completely barley-based with no additional protein source, YEA = barley-based with additional protein from yeast (*C. jadinii*))

FA	SBM	BAR	YEA	Grass silage
C6:0	0.10 ± 0.01	$0.11 \pm < 0.01$	0.10 ± 0.01	$0.02 \pm < 0.01$
C8:0	$0.08 \pm < 0.01$	$0.09 \pm < 0.01$	$0.06 \pm < 0.01$	$0.03 \pm < 0.01$
C9:0	$0.10 \pm < 0.01$	$0.11 \pm < 0.01$	$0.06 \pm < 0.01$	$0.02 \pm < 0.01$
C10:0	$0.02 \pm < 0.01$	$0.02 \pm < 0.01$	$0.02 \pm < 0.01$	$0.03 \pm < 0.01$
C12:0	$0.10 \pm < 0.01$	$0.11 \pm < 0.01$	$0.10 \pm < 0.01$	$0.07 \pm < 0.01$
C14:0	0.93 ± 0.01	0.95 ± 0.02	0.80 ± 0.03	0.35 ± 0.01
C15:0	$0.11 \pm < 0.01$	$0.11 \pm < 0.01$	$0.13 \pm < 0.01$	$0.15 \pm < 0.01$
C16:0	52.26 ± 0.31	52.97 ± 0.12	47.29 ± 0.52	22.88 ± 0.03
C16:1*n-9cis*7	$0.03 \pm < 0.01$	$0.02 \pm < 0.01$	$0.03 \pm < 0.01$	$0.04 \pm < 0.01$
C16:1*n-7cis9*	$0.13 \pm < 0.01$	$0.13 \pm < 0.01$	0.28 ± 0.01	2.16 ± 0.01
Anteiso-C16:0	$0.01 \pm < 0.01$	$0.01 \pm < 0.01$	$0.01 \pm < 0.01$	$0.06 \pm < 0.01$
C17:0	$0.17 \pm < 0.01$	$0.16 \pm < 0.01$	$0.20 \pm < 0.01$	0.20 ± 0.01
C17:1*n-8cis*9	$0.02 \pm < 0.01$	$0.02 \pm < 0.01$	0.13 ± 0.01	$0.03 \pm < 0.01$
C18:0	5.83 ± 0.03	$5.71 \pm < 0.01$	5.28 ± 0.08	1.53 ± 0.01
$\sum$C18:1[1]	$0.10 \pm < 0.01$	0.09 ± 0.02	0.13 ± 0.01	n.d.[3]
C18:1*n-9cis*9	29.07 ± 0.15	29.30 ± 0.17	32.19 ± 0.07	3.26 ± 0.09
C18:1n-7*cis*11	0.87 ± 0.01	0.84 ± 0.02	$0.87 \pm < 0.01$	$0.48 \pm < 0.01$
C18:2*n-6cis*9,12	8.32 ± 0.3	7.66 ± 0.13	10.53 ± 0.65	16.28 ± 0.27

C19:1[1]	n.d.[3]	n.d.[3]	n.d.[3]	0.11 ± 0.01
C18:3[1]	n.d.[3]	n.d.[3]	n.d.[3]	0.17 ± 0.01
C20:0	0.43 ± 0.01	$0.40 \pm < 0.01$	0.36 ± 0.01	n.d.[3]
C18:3n-3cis9,12,15	0.31 ± 0.01	0.26 ± 0.01	0.39 ± 0.02	49.02 ± 0.27
C20:1n-9cis11	0.35 ± 0.01	$0.35 \pm < 0.01$	0.4 ± 0.01	0.77 ± 0.06
C18:2[1]	0.20 ± 0.01	0.17 ± 0.03	0.18 ± 0.01	$0.02 \pm < 0.01$
C21:0	n.d.[3]	n.d.[3]	n.d.[3]	$0.03 \pm < 0.01$
C20:2n-6cis11,14	n.d.[3]	n.d.[3]	n.d.[3]	$0.06 \pm < 0.01$
C22:0	$0.19 \pm < 0.01$	$0.16 \pm < 0.01$	0.16 ± 0.01	0.60 ± 0.03
C22:1n-9cis13	0.03 ± 0.01	$0.02 \pm < 0.01$	$0.03 \pm < 0.01$	n.d.[3]
C23:0	$0.04 \pm < 0.01$	$0.03 \pm < 0.01$	$0.03 \pm < 0.01$	$0.06 \pm < 0.01$
C24:0	$0.15 \pm < 0.01$	$0.13 \pm < 0.01$	$0.13 \pm < 0.01$	0.33 ± 0.02
C24:1n-9cis15	$0.02 \pm < 0.01$	$0.01 \pm < 0.01$	$0.02 \pm < 0.01$	n.d.[3]
C26:0	$0.06 \pm < 0.01$	$0.05 \pm < 0.01$	$0.08 \pm < 0.01$	0.41 ± 0.05
C28:0[2]	n.d.[3]	n.d.[3]	n.d.[3]	0.57 ± 0.06
C30:0[2]	n.d.[3]	n.d.[3]	n.d.[3]	0.27 ± 0.04

198   [1]Non-identified isomer.

199   [2]Identified using library search.

200   [3]n.d.: not detected.

201 Supplementary table S2. The fatty acid (FA) composition in milk (g/100g of FAs) from cows

202 fed grass silage augmented with three different concentrate feeds containing additional

203 protein from either soybean meal (SBM), barley (BAR) or yeast (YEA) over a period of 10

204 weeks.

FA	Feed	Control period[1] (Week)	Experimental period (Week)		SEM[2]
		2	6	10	
C4:0	SBM	0.281	0.274	0.279	0.004
	BAR	0.302	0.289	0.278	
	YEA	0.276	0.268	0.259	
	SEM[3]	0.007			
C6:0	SBM	0.549	0.529	0.533	0.007
	BAR	0.568	0.545	0.523	
	YEA	0.532	0.511	0.503	
	SEM[3]	0.010			
C7:0	SBM	0.011	0.010	0.010	<0.001
	BAR	0.010	0.011	0.010	
	YEA	0.011	0.010	0.009	
	SEM[3]	<0.001			
C8:0	SBM	0.509	0.489	0.486	0.006
	BAR	0.515	0.489	0.475	
	YEA	0.493	0.475	0.469	
	SEM[3]	0.008			
C9:0	SBM	0.017	0.015	0.015	<0.001
	BAR	0.016	0.015	0.015	
	YEA	0.015	0.014	0.014	
	SEM[3]	0.001			
C10:0	SBM	1.788	1.705	1.716	0.023
	BAR	1.747	1.686	1.619	
	YEA	1.748	1.678	1.693	
	SEM[3]	0.027			
C10:1n-6$trans$4[4]	SBM	0.144	0.144	0.148	0.003
	BAR	0.140	0.136	0.131	
	YEA	0.138	0.145	0.134	
	SEM[3]	0.004			
C12:0	SBM	2.807	2.694	2.682	0.033
	BAR	2.684	2.576	2.537	
	YEA	2.754	2.668	2.694	
	SEM[3]	0.041			
Iso-C12:0	SBM	0.017	0.018	0.018	<0.001
	BAR	0.015	0.015	0.016	
	YEA	0.017	0.018	0.018	

	SEM[3]	<0.001			
C12:1n-7cis5[4]	SBM	0.059	0.059	0.059	0.001
	BAR	0.051	0.052	0.052	
	YEA	0.056	0.059	0.054	
	SEM[3]	0.002			
Anteiso-C12:0[4]	SBM	0.006	0.006	0.006	<0.001
	BAR	0.005	0.006	0.006	
	YEA	0.006	0.006	0.006	
	SEM[3]	<0.001			
C9:0-cyclopropane[4]	SBM	0.062	0.061	0.063	0.001
	BAR	0.055	0.055	0.052	
	YEA	0.061	0.062	0.060	
	SEM[3]	0.002			
C13:0	SBM	0.082	0.077	0.078	0.002
	BAR	0.082	0.077	0.075	
	YEA	0.079	0.075	0.076	
	SEM[3]	0.002			
Iso-C13:0	SBM	0.079	0.078	0.076	0.001
	BAR	0.073	0.077	0.076	
	YEA	0.086	0.076	0.080	
	SEM[3]	0.002			
C14:0	SBM	10.62	10.38	10.35	0.07
	BAR	10.47	10.25	10.08	
	YEA	10.62	10.43	10.44	
	SEM[3]	0.10			
Iso-C14:0	SBM	0.229	0.233	0.240	0.003
	BAR	0.220	0.217	0.225	
	YEA	0.236	0.235	0.241	
	SEM[3]	0.003			
C14:1n-5cis9	SBM	1.262	1.315	1.358	0.023
	BAR	1.203	1.223	1.211	
	YEA	1.300	1.397	1.320	
	SEM[3]	0.030			
C14:1n-3cis11[4]	SBM	0.042	0.036	0.054	0.004
	BAR	0.031	0.047	0.028	
	YEA	0.041	0.039	0.036	
	SEM[3]	0.002			
C15:0	SBM	1.158	1.182	1.238	0.013
	BAR	1.141	1.146	1.184	
	YEA	1.140	1.180	1.198	
	SEM[3]	0.017			
∑C15:1[5a)]	SBM	0.054	0.056	0.054	0.001
	BAR	0.056	0.059	0.057	
	YEA	0.057	0.056	0.056	
	SEM[3]	0.001			
Iso-C15:0	SBM	0.222	0.205	0.196	0.003

	BAR	0.206	0.214	0.204	
	YEA	0.245	0.206	0.200	
	SEM[3]	0.006			
C16:0	SBM	37.80	37.31	37.42	0.22
	BAR	37.80	37.36	37.84	
	YEA	36.89	36.37	36.32	
	SEM[3]	0.23			
C16:1n-7$trans$9	SBM	0.065	0.064	0.062	0.001
	BAR	0.062	0.061	0.064	
	YEA	0.068	0.064	0.062	
	SEM[3]	0.002			
C16:1n-9cis7	SBM	0.127	0.123	0.125	0.002
	BAR	0.129	0.134	0.136	
	YEA	0.132	0.126	0.124	
	SEM[3]	0.002			
C16:1n-7cis9	SBM	1.711	1.798	1.900	0.038
	BAR	1.831	1.889	1.957	
	YEA	1.826	1.953	1.932	
	SEM[3]	0.052			
$Anteiso$-C16:0	SBM	0.424	0.434	0.418	0.005
	BAR	0.415	0.428	0.413	
	YEA	0.428	0.429	0.435	
	SEM[3]	0.007			
C16:1[5]	SBM	0.216	0.214	0.216	0.005
	BAR	0.176	0.175	0.176	
	YEA	0.207	0.219	0.206	
	SEM[3]	0.007			
C17:0	SBM	0.596	0.697	0.622	0.014
	BAR	0.521	0.619	0.597	
	YEA	0.579	0.685	0.715	
	SEM[3]	0.013			
C16:0-3,7,11,15-tetramethyl	SBM	0.145	0.141	0.182	0.008
	BAR	0.150	0.130	0.193	
	YEA	0.120	0.138	0.170	
	SEM[3]	0.008			
C17:1[5]	SBM	0.089	0.096	0.097	0.002
	BAR	0.080	0.093	0.104	
	YEA	0.092	0.095	0.103	
	SEM[3]	0.002			
C17:1n-8cis9	SBM	0.133	0.145	0.153	0.002
	BAR	0.131	0.144	0.154	
	YEA	0.138	0.166	0.163	
	SEM[3]	0.003			
Iso-C17:0	SBM	0.028	0.030	0.028	0.001
	BAR	0.023	0.025	0.029	
	YEA	0.027	0.025	0.027	

	SEM[3]	0.001			
C18:0	SBM	12.90	12.68	12.23	0.13
	BAR	12.59	12.42	12.47	
	YEA	12.87	12.01	12.75	
	SEM[3]	0.20			
∑C18:1[5b)]	SBM	0.768	0.715	0.693	0.012
	BAR	0.760	0.749	0.759	
	YEA	0.767	0.689	0.707	
	SEM[3]	0.015			
C18:1n-9$trans$9	SBM	0.302	0.271	0.243	0.005
	BAR	0.326	0.284	0.288	
	YEA	0.313	0.285	0.267	
	SEM[3]	0.008			
C18:1n-7$trans$11	SBM	1.395	1.444	1.392	0.030
	BAR	1.491	1.503	1.400	
	YEA	1.474	1.552	1.425	
	SEM[3]	0.042			
C18:1n-9cis9	SBM	19.44	20.29	20.78	0.18
	BAR	19.92	20.66	20.61	
	YEA	20.14	21.41	21.11	
	SEM[3]	0.21			
C18:1n-7cis11	SBM	0.692	0.644	0.639	0.009
	BAR	0.682	0.677	0.680	
	YEA	0.675	0.612	0.649	
	SEM[3]	0.013			
C18:1n-5$trans$13[4]	SBM	0.279	0.268	0.257	0.004
	BAR	0.304	0.282	0.278	
	YEA	0.289	0.294	0.259	
	SEM[3]	0.008			
C18:1n-5cis13	SBM	0.020	0.018	0.020	<0.001
	BAR	0.019	0.019	0.021	
	YEA	0.022	0.021	0.021	
	SEM[3]	0.001			
C11:0-cyclohexyl[4]	SBM	0.159	0.150	0.144	0.003
	BAR	0.165	0.169	0.159	
	YEA	0.165	0.157	0.144	
	SEM[3]	0.004			
∑C18:2[5c)]	SBM	0.207	0.241	0.246	0.009
	BAR	0.220	0.246	0.250	
	YEA	0.229	0.250	0.239	
	SEM[3]	0.007			
C18:2n-6cis9,12	SBM	1.136	1.106	0.991	0.016
	BAR	1.239	1.201	1.057	
	YEA	1.204	1.186	1.065	
	SEM[3]	0.025			
C19:1n-9cis10	SBM	0.035	0.039	0.042	0.001

	BAR	0.035	0.041	0.040	
	YEA	0.037	0.042	0.045	
	SEM[3]	0.001			
C19:1[4]	SBM	0.017	0.022	0.023	0.001
	BAR	0.016	0.022	0.020	
	YEA	0.018	0.018	0.023	
	SEM[3]	0.001			
C20:0	SBM	0.118	0.139	0.118	0.005
	BAR	0.118	0.142	0.119	
	YEA	0.108	0.124	0.115	
	SEM[3]	0.006			
C18:3n-3cis9,12,15	SBM	0.313	0.335	0.339	0.006
	BAR	0.306	0.328	0.350	
	YEA	0.330	0.358	0.358	
	SEM[3]	0.007			
C18:2n-7cis9trans11	SBM	0.526	0.649	0.626	0.015
	BAR	0.562	0.626	0.613	
	YEA	0.575	0.726	0.655	
	SEM[3]	0.013			
C20:1n-9cis11	SBM	0.031	0.027	0.027	0.001
	BAR	0.030	0.029	0.029	
	YEA	0.032	0.031	0.026	
	SEM[3]	0.001			
C21:0	SBM	0.033	0.040	0.031	0.001
	BAR	0.033	0.043	0.037	
	YEA	0.031	0.040	0.034	
	SEM[3]	0.001			
C18:3[5]	SBM	0.036	0.032	0.026	0.002
	BAR	0.028	0.027	0.031	
	YEA	0.032	0.043	0.029	
	SEM[3]	0.002			
C20:3n-6cis8,11,14	SBM	0.048	0.049	0.038	0.001
	BAR	0.043	0.049	0.046	
	YEA	0.046	0.046	0.044	
	SEM[3]	0.002			
C22:0	SBM	0.047	0.046	0.045	0.001
	BAR	0.049	0.051	0.051	
	YEA	0.046	0.044	0.045	
	SEM[3]	0.002			
C20:4n-6 cis5,8,11,14	SBM	0.042	0.043	0.034	0.001
	BAR	0.038	0.043	0.040	
	YEA	0.044	0.046	0.039	
	SEM[3]	0.002			
C20:4n-3cis8,11,14,17	SBM	0.017	0.017	0.019	0.001
	BAR	0.014	0.014	0.018	
	YEA	0.016	0.016	0.018	

	SEM[3]	0.001			
C20:5n-3cis5,8,11,14,17	SBM	0.019	0.021	0.020	0.001
	BAR	0.021	0.022	0.025	
	YEA	0.020	0.021	0.020	
	SEM[3]	0.001			
C23:0	SBM	0.011	0.013	0.012	0.001
	BAR	0.011	0.012	0.015	
	YEA	0.011	0.012	0.014	
	SEM[3]	0.001			
C23:1[4]	SBM	0.031	0.032	0.032	0.001
	BAR	0.028	0.031	0.034	
	YEA	0.032	0.030	0.033	
	SEM[3]	0.001			
C24:0	SBM	0.012	0.014	0.013	<0.001
	BAR	0.012	0.013	0.014	
	YEA	0.012	0.012	0.011	
	SEM[3]	<0.001			
C25:5n-6cis7,10,13,16,19[4]	SBM	0.039	0.047	0.041	0.002
	BAR	0.032	0.046	0.039	
	YEA	0.041	0.039	0.039	
	SEM[3]	0.002			

205 [1]All cows were fed the same SBM concentrate for two weeks in the control period.

206 [2]Standard error of the mean for the experimental period.

207 [3]Standard error of the mean for the control period.

208 [4]Identified using library search.

209 [5]Non-identified isomers:

210   a) Sum of 2 isomers.

211   b) Sum of 3 isomers.

212   c) Sum of 4 isomers.

213 Supplementary table S3. The amount of fatty acids (FAs; g/100g of FAs) in milk from cows

214 fed grass silage augmented with three different concentrates (SBM = barley-based with

215 additional protein from soybean meal, BAR = completely barley-based with no additional

216 protein source, YEA = barley-based with additional protein from yeast (*C. jadinii*)) showing

217 a significant effect (p<0.05) of the concentrate or of the experimental week. P-values were

218 calculated using values adjusted for covariates and values from the SBM-fed group as

219 reference.

FA	Feed	Control period[1] (Week) 2	Experimental period (Week) 6	10	SEM[2]	BAR	YEA	Week
Iso-C14:0	SBM	0.229	0.233	0.240	0.003	0.32	0.50	0.03
	BAR	0.220	0.217	0.225				
	YEA	0.236	0.235	0.241				
	SEM[3]	0.003						
C15:0	SBM	1.158	1.182	1.238	0.013	0.43	0.60	0.01
	BAR	1.141	1.146	1.184				
	YEA	1.140	1.180	1.198				
	SEM[3]	0.017						
Iso-C15:0	SBM	0.222	0.205	0.196	0.003	0.03	0.12	0.09
	BAR	0.206	0.214	0.204				
	YEA	0.245	0.206	0.200				
	SEM[3]	0.006						
C16:1*n*-9*cis*7	SBM	0.127	0.123	0.125	0.002	0.04	0.53	0.70
	BAR	0.129	0.134	0.136				
	YEA	0.132	0.126	0.124				
	SEM[3]	0.002						
C16:1*n*-7*cis*9	SBM	1.711	1.798	1.900	0.038	0.35	0.59	0.02
	BAR	1.831	1.889	1.957				
	YEA	1.826	1.953	1.932				

					SEM[3]			
	SEM[3]	0.052						
C16:0-3,7,11,15-tetramethyl	SBM	0.145	0.141	0.182	0.008	0.80	0.54	<0.01
	BAR	0.150	0.130	0.193				
	YEA	0.120	0.138	0.170				
	SEM[3]	0.008						
C17:1[5]	SBM	0.089	0.096	0.097	0.002	0.02	0.85	0.03
	BAR	0.080	0.093	0.104				
	YEA	0.092	0.095	0.103				
	SEM[3]	0.002						
C17:1n-8cis9	SBM	0.133	0.145	0.153	0.002	0.38	0.01	0.07
	BAR	0.131	0.144	0.154				
	YEA	0.138	0.166	0.163				
	SEM[3]	0.003						
C18:1n-7trans11	SBM	1.395	1.444	1.392	0.030	0.23	0.72	0.02
	BAR	1.491	1.503	1.400				
	YEA	1.474	1.552	1.425				
	SEM[3]	0.042						
C18:1n-7cis11	SBM	0.692	0.644	0.639	0.009	0.04	0.83	0.17
	BAR	0.682	0.677	0.680				
	YEA	0.675	0.612	0.649				
	SEM[3]	0.013						
C18:1n-5trans13	SBM	0.279	0.268	0.257	0.004	0.55	0.91	0.02
	BAR	0.304	0.282	0.278				
	YEA	0.289	0.294	0.259				
	SEM[3]	0.008						
C11:0-cyclohexyl[4]	SBM	0.159	0.150	0.144	0.003	0.17	0.54	0.04
	BAR	0.165	0.169	0.159				
	YEA	0.165	0.157	0.144				
	SEM[3]	0.004						
C18:1[5]	SBM	0.361	0.344	0.343	0.005	0.02	0.75	0.07
	BAR	0.346	0.350	0.365				
	YEA	0.352	0.329	0.347				

	SEM[3]	0.006						
C18:2n-6cis9,12	SBM	1.136	1.106	0.991	0.016	0.52	0.87	<0.01
	BAR	1.239	1.201	1.057				
	YEA	1.204	1.186	1.065				
	SEM[3]	0.025						
C18:2n-7cis9trans11	SBM	0.526	0.649	0.626	0.015	0.14	0.84	0.01
	BAR	0.562	0.626	0.613				
	YEA	0.575	0.726	0.655				
	SEM[3]	0.013						
C21:0	SBM	0.033	0.040	0.031	0.001	0.40	0.45	<0.01
	BAR	0.033	0.043	0.037				
	YEA	0.031	0.040	0.034				
	SEM[3]	0.001						
C18:3[5]	SBM	0.036	0.032	0.026	0.002	0.07	0.02	0.08
	BAR	0.028	0.027	0.031				
	YEA	0.032	0.043	0.029				
	SEM[3]	0.002						
C20:3n-6cis8,11,14	SBM	0.048	0.049	0.038	0.001	0.01	0.21	<0.01
	BAR	0.043	0.049	0.046				
	YEA	0.046	0.046	0.044				
	SEM[3]	0.002						
C20:4n-6cis5,8,11,14	SBM	0.042	0.043	0.034	0.001	0.05	0.49	<0.01
	BAR	0.038	0.043	0.040				
	YEA	0.044	0.046	0.039				
	SEM[3]	0.002						
C20:4n-3cis8,11,14,17	SBM	0.017	0.017	0.019	0.001	0.51	0.99	<0.01
	BAR	0.014	0.014	0.018				
	YEA	0.016	0.016	0.018				
	SEM[3]	0.001						

220 [1]All cows were fed the SBM concentrate for two weeks in the control period.

221 [2]Standard error of the mean for the experimental period.

222   [3]Standard error of the mean for the control period.

223   [4]Identified using library search.

224   [5]Non-identified isomer.

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