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Dietary supplementation of dairy cows with a docosahexaenoic acid-rich thraustochytrid, *Aurantiochytrium limacinum*: effects on milk quality, fatty acid composition and cheese making properties

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ABSTRACT. The aim of this study was to evaluate the effect of heterotrophically grown docosahexaenoic acid (DHA)-rich thraustochytrid *Aurantiochytrium limacinum* (AURA) added to dairy cow diet on milk fatty acid profile and milk cheese making properties. The secondary aim was to investigate the effect of pelleting on DHA transfer from diet to milk. Thirty six lactating dairy cows were blocked by parity, number of days in milk and by milk production and randomly assigned to 1 of 3 diets: AURA (150 g/cow/day) with protein concentrate in pelleted or meal form, and control group – protein concentrate in pelleted form without AURA. Milk samples from each cow were taken on days 0, 28, 56 and 84. Dietary supplementation for 84 days resulted in the successful enrichment of milk with DHA, at a level of 4.47 and 6.37 mg/100 ml milk for the groups supplemented with AURA in a pelleted and meal form, respectively. As less DHA was detected in the pelleted concentrate (470 vs 570 mg/kg), and subsequently in the milk of the groups fed these pellets, the process of pelleting may have resulted in a loss of DHA from the feed. Dietary supplementation with AURA improved milk quality: increased DHA content and lowered n-6:n-3 ratio. With no differences observed for the cheese making properties of milk from cows fed supplemented or control diets, it can be stated that milk obtained from cows fed diet enriched with DHA-rich thraustochytrid *Aurantiochytrium limacinum* at a dose 150 g/cow/day (which gives about 24 g DHA/cow/day) can be suitable for cheese production.

Introduction

Polyunsaturated fatty acids (PUFA) are considered essential for general health and wellbeing (Gómez Candela et al., 2011), and may also play a role in limiting the effects of a number of chronic diseases (Ruxton et al., 2014). Omega-3 (n-3) and omega-6 (n-6) PUFA are both required to maintain good health; however, in many western countries n-3 fatty acid (FA) intake is below recommended levels, while n-6 PUFA intake is often considerably above recommended levels (Simopoulos, 2008). To address the lack of n-3 PUFA available in the food chain, dietary supplementation of intensively farmed animals with microalgae has been successfully used to increase the n-3 PUFA content in animal products (Moran et al., 2018a,b,c; 2019). For ruminants, the alteration of unsaturated fatty acids to form saturated fatty acids by ruminal microbes, *via* the processes of lipolysis and biohydro-

genation, has made enrichment more difficult (Zymon et al., 2014). Despite these challenges, ruminant milk and meat have been successfully enriched (Papadopoulos et al., 2002; Moate et al., 2013; Moran et al., 2018b).

Few studies have investigated the effect of feeding dairy cows docosahexaenoic acid (DHA)-rich marine protists, such as microalgae or thraustochytrids, on the cheese making properties of milk. Dietary supplementation of cows with plant and fish oils, has been shown to enhance the PUFA content in milk and cheese without negatively affecting their consumer acceptability (Jones et al., 2005; Allred et al., 2006; Caroprese et al., 2013). Avramis et al. (2003) reported that cheddar cheese made from the milk of cows fed diets supplemented with fish meal ripened faster and developed a stronger flavour and texture. However, differences in casein micelle size and composition between the control and treatment groups, and a slower rate of firming when using the milk of cows fed fish meal supplemented diets, indicated potential difficulties in terms of cheese production.

Few studies have investigated the effect of pelleting on the bioavailability of DHA in dairy cows. A previous study on chickens indicated that pelleting does not affect the transfer of DHA into eggs (Moran et al., 2017a); however, the effect of pelleting on the transfer of DHA from the diet into milk remains to be evaluated.

So, the primary aim of this study was to evaluate the effect of enrichment of dairy cows diet with a heterotrophically grown DHA-rich thraustochytrid, *Aurantiochytrium limacinum* (AURA), on milk quality and its fatty acid profile, and milk cheese making properties. The secondary aim was to investigate the effect of pelleting on DHA transfer and bioavailability from diet.

Material and methods

Animals, experimental design and diets

The study was carried out at the CERZOO S.r.L. Research Centre (Piacenza, Italy) in compliance with G.L.P guidelines (Directives 2004/9/EC and 2004/10/EC) for the collection, handling and documentation of data. The research protocol and animal care were carried out in accordance with European guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU). Cows were kept in six pens of six animals each on straw and chip litter bedding. Pens were cleaned and the bedding was renewed weekly. Animals were checked daily to ensure they were fit to take part in the trial and provided with free access to water. The cows were milked twice a day in a room with milking stalls equipped with Afimilk (Afimilk Ltd., Kibbutz Afikim, Israel) control panels which enabled individual identification of each cow facilitating the recording of production data.

Thirty-six lactating, Italian Friesian dairy cows [parity 2.3 ± 1.3; weight 672 ± 73 kg; days in milk (DIM) 164 ± 65; milk yield 39 ± 8 kg] were selected from those present on the farm to provide a group representative of various lactation stages. The cows were acclimatised to these conditions for a period of 10 days while under daily veterinary supervision to ensure they were fit to take part in the study. Prior to enrolment in the study, the cows were blocked by parity, number of DIM and milk production before being randomly assigned to 1 of 3 isonitrogenous and isoenergetic total mixed rations (TMR). Diets were formulated to meet or exceed the requirements of the average cow in the group according to the nutrient requirements of dairy cattle of National Reseach Council (NRC, 2001). The TMR contained maize silage (26.0 kg), protein concentrate (4.9 kg), hydrogenated palm oil (0.32 kg), maize and barley flake mix (60:40 ratio, 1.5 kg), maize and sorghum meal mix (80:20 ratio, 4.8 kg), rye grass hay (1.1 kg), dehydrated alfalfa hay (4.5 kg) and water (3.0 kg). Minerals and vitamins were provided with the protein concentrate mixture. The protein concentrate composition for the control and treatment groups is shown in Table 1. The TMRs were supplemented through the protein concentrate, with 150 g/cow/day of AURA provided

Table 1. Composition of the concentrates used in the preparation of the total mixed ration (TMR) for the control and *Aurantiochytrium limacinum* (AURA[1]) supplemented groups (% dry matter, DM)

Concentrate composition	Control, % DM	Treatment, % DM
Soybean meal	44.34	44.43
Sunflower dehulled meal	18.5	18.0
Corn gluten meal	18.5	18.0
Flaked soybeans	6.0	6.0
Sodium bicarbonate	3.4	3.4
Calcium carbonate	3.4	3.4
Hydrogenated palm oil fat	2.16	0
Magnesium oxide	1.2	1.2
Dicalcium phosphate	0.8	0.8
Sodium chloride	0.8	0.8
Premix[2]	0.8	0.8
Zinc sulphate	0.1	0.1
AURA	0	3.07

[1] AURA – unextracted *Aurantiochytrium limacinum* algae containing 17.04 g DHA/100 g (Alltech Inc., Nicholasville, KY, USA); [2] each kg of premix contained: IU: vit. A 47 640, vit. D_3 4 368; mg: vit. E 85.68, Ca 13.47, P 6.02, Mg 4.45, Na 12.65, Cu 47.35, Fe 261.16, Zn 135.90, I 1.55, Co 16.67, Mn 138.65, Se 0.47

by Alltech Inc. (ALL-G-RICH®, Nicholasville, KY, USA) and consisted of a heterotrophically grown, unextracted *Aurantiochytrium limacinum* (CCAP 4087/2) biomass for groups with AURA in a pelleted protein concentrate (APC) or AURA in a meal protein concentrate (AMC). These treated groups were compared to the control group with a pelleted protein concentrate (CPC). The protein concentrates for CPC, APC and AMC groups, were produced by a single mixing at Luigi Ferrari Feed mill (Sarmato, Piacenza, Italy), where the following conditions were adopted for the pelleted protein concentrate for CPC and APC groups production: vapour conditioning and pelleting (temperature 70–75 °C), pellet with diameter of 5 mm and length of 12–15 mm.

The TMR was provided using one steel feeder per pen, with TMR intake recorded per pen and divided by the number of animals per pen to achieve an estimation of mean intake per cow. The composition of the three different TMRs was checked every 28 days. The TMRs were mixed in a Labrador 70 wagon (Storti, Belfiore, Verona, Italy), with the control TMR being mixed before the treatment TMRs to avoid cross-contamination. After the initial 10-day acclimatisation period, the groups were assigned and their feed and various productivity measurements were recorded over a period of 84 days. Intake of AURA was calculated based on the level of inclusion of the thraustochytrid in the treated protein concentrates (3.07%) and the amount of protein concentrate in the TMR (4.9 kg/cow/day = 10.62%). The mean AURA intake for groups APC and AMC was then calculated using the mean TMR intake of each treated group for the entire period of the study that the algae was included in the feed (days 1–84).

Sampling, measurements and analysis

Fatty acid composition (method no. 996.06, AOAC International (2005); official method no. Ce 2c-11, AOCS (2017)) and crude fat content (method no. 954.02, AOAC International (2005)) of the AURA supplement were determined prior to the start of the study at Eurofins Scientific Inc. (Des Moines, IA, USA). Further analytical tests of the AURA supplement were performed at Chelab S.r.l, (Resana, Italy) using methods described in Regulation EC 152/2009 (Annex III): crude protein (Method C), crude fibre (Method I), moisture (Method A) and ash (Method M). In order to ensure the ration met the requirements of the trial animals, the nutrient composition of fresh TMR samples were analysed four times (every 28 days) using standardised techniques: crude protein (ISO 5983-1), ADF (ISO 13906), NDF (ISO 16472), starch (ISO 10520:1997E), crude fat (ISO 6492), non-fibre carbohydrates – NFC; calculated according to equation: 100 − [CP + ash + EE + (NDF − NDICP)], predicted metabolizable energy and net energy lactation (Gallo et al., 2013). Dry matter was calculated weekly by force drying TMR samples at 103 °C to a constant weight (ISO 6496). The DHA content of the concentrate and TMR samples was quantified (every 28 days) by direct fatty acid methyl ester synthesis according to O'Fallon et al. (2007), followed by quantification using the gas chromatography method described by Bannon et al. (1985).

Health/Performance

Live weight (LW) was recorded per cow, twice daily from day 0 to 84 and was calculated as the mean of the weights recorded automatically after milking. Individual milk production was also recorded from day 0 to 84 for each animal as the sum of the morning and afternoon milkings. Group TMR intake (dry matter basis) was calculated daily based on the total TMR offered per pen minus that refused. Body condition scores (BCS) were recorded on days 0 and 84, by the same person using a score on a 0 to 5 scale, with one-point increments according to Agricultural Development and Advisory Service (ADAS, 1986). The rumination activity of all cows (n = 36) was recorded continuously using acoustic sensors (RuminACT, Milkline, Piacenza, Italy) for the entire 84-day treatment period, with data analysed at the Institute of Zootechnics, Catholic University of the Sacred Heart (Piacenza, Italy).

Milk yield and composition

Analysis of the milk was carried out for each cow on days 0, 28, 56 and 84. The milk sampling was carried out as follows: milk samples were taken from each cow in the morning and afternoon on two consecutive days; 5% of the milk production from each of the four milkings were then combined to generate a pool for each cow; the pool was then divided into three aliquots of at least 50 ml each. For each cow, the first aliquot was used for the analysis of milk components (i.e. fat, protein, lactose, urea, somatic cell count). The second milk aliquot was used for the analysis of cheese making qualities (e.g., coagulant property, fermentative aptitude, natural creaming). Fat corrected milk (FCM, 4%) was calculated as per Gaines and Davidson's formula (1923): FCM (kg) = 0.4M + 15F where: M – milk yield (kg) and F – M × fat content (%).

The third aliquot was used to establish the milk fatty acid (FA) profile, by direct fatty acid methyl ester synthesis according to O'Fallon et al. (2007), followed by quantification using the gas chromatography method described by Bannon et al. (1985). DHA transfer efficiency from diet to milk was calculated as: DHA in milk yield (g/d) / DHA intake (g/d) × 100.

Milk cheese making properties

The cheese making qualities were established using the following methods:

- milk casein: Fourier transform mid-infrared spectroscopy (FT-MIR with Milkoscan FT-120; Foss Electric, Hillerød, Denmark);
- milk titratable acidity: MicroTT 2050 (Crison, Barcelona, Spain) automated titration system;
- milk fat natural creaming: method according to Speroni and Bertoni (1984);
- milk rennet coagulation: assessed as clotting time (r30), curd forming rate (k20) and curd firmness (a30) using the Formagraph (Foss Electric, Hillerød, Denmark) where 10 ml milk was heated to 35 °C, and 200 µl rennet (Hansen standard 190 with 63% chymosin and 37% pepsin; Pacovis rein, Bern, Switzerland) diluted to 1.6% (w/w) in distilled water was added to milk.

Statistical analysis

For milk yield, LW, milk fatty acid profile and milk quality parameters the individual cow was the experimental unit. Results were checked for normality using the Shapiro-Wilk test and data were then analysed as repeated measures in a randomized design using the MIXED procedure of SAS (release 9.3, 2002–2010; SAS Institute Inc., Cary, NC, USA). Measured variables were subjected to two covariance structures: compound symmetry and autoregressive. The Akaike information criterion and the Schwarz Bayesian criterion were used to find out the covariance structures that best fit to the model for each parameter with significance being declared at $P \leq 0.05$. Data concerning milk parameters were also analysed using the General Linear Model (GLM) procedure of SAS. Tukey test was used to compare the means of each group. As the BCS data was not normally distributed the non-parametric Kruskal-Wallis test was used to establish the effect of AURA supplementation on BCS. Significant differences were indicated at $P \leq 0.05$. When significant differences were observed at day 0, these values were used as a covariate in the overall analysis.

Results

Ingredient and diet analysis

The AURA used in the study contained 70.2 g of crude fat/100 g dry matter (DM) biomass and 17.0 g DHA/100 g DM biomass with a significant amount of palmitic acid (36.0 g/100 g DM biomass). Additionally, the AURA contained 13.1% crude protein, 3.2% ash and 2.2% moisture. The nutrient composition of the TMR over the course of the experiment is summarised for the three groups (CPC, APC and AMC) in Table 2. Based on the mean pen TMR intake the estimated AURA intake for the APC and AMC groups was 142 and 144 g/cow/day, respectively, which would provide 24.14 and 24.48 g DHA/cow/day. The mean DHA content detected in the TMR was 0.47 and 0.57 g/kg for the APC and AMC groups, respectively. Based on the calculated TMR intake of the experimental groups, the mean estimated intake of DHA was 20.4 and 25.1 g/cow/day for the APC and AMC groups, respectively.

Table 2. Analytical composition (dry matter basis) (mean ± S.D.) of control (CP) and *Aurantiochytrium limacinum* (AURA[1]) supplemented total mixed rations (TMR)

Indices	Control pellet	AURA pellet	AURA meal
Dry matter (DM), %	51.85 ± 0.21	51.66 ± 0.24	51.59 ± 0.38
Crude protein, % DM	15.43 ± 0.01	15.27 ± 0.06	15.28 ± 0.18
Fat, % DM	5.00 ± 0.07	4.97 ± 0.10	4.96 ± 0.12
Non-fibre carbohydrates, % DM	36.29 ± 0.58	36.37 ± 0.42	36.13 ± 0.27
ADF, % DM	22.20 ± 0.28	22.69 ± 0.17	22.41 ± 0.27
NDF, % DM	37.25 ± 0.52	37.32 ± 0.40	37.48 ± 0.15
Starch, % DM	29.04 ± 0.88	29.22 ± 0.45	29.35 ± 0.36
Ash, % DM	6.08 ± 0.14	6.08 ± 0.11	6.15 ± 0.10
Metabolizable energy, Mcal/kg	2.69 ± 0.01	2.69 ± 0.01	2.68 ± 0.01
Net energy lactation, Mcal/kg	1.71 ± 0.01	1.70 ± 0.01	1.70 ± 0.01
Docosahexaenoic acid (DHA), mg/kg	0	470 ± 0.01	570 ± 0.01

[1] AURA – unextracted *Aurantiochytrium limacinum* containing 17.04 g DHA/100 g (Alltech Inc., Nicholasville, KY, USA)

Animal productivity and milk parameters

No differences in BCS, LW, rumen activity or milk yield (Table 3; Figure 1) were observed between the groups over the course of the study. Although there were no significant differences between the groups in terms of the milk SCC, a nu-

Table 3. Performance indicators and milk quality parameters of cows fed total mixed rations (TMR) in the form of control pellet (CPC) or *Aurantiochytrium limacinum* (AURA[1]) supplemented pellet (APC) or meal (AMC) during an 84-day trial[2]

Parameter	Control pellet	AURA pellet	AURA meal	S.E.M.[8]	*P*-value
Body condition score[3]	2.36	2.35	2.34	0.10	0.99
TMR intake[4,5], kg DM	22.73	22.53	22.73	0.03	0.21
Body weight (mean per cow)[6], kg	663	663	668	21.57	0.99
Rumination activity, min/cow/day	486	456	443	18.65	0.27
Milk yield[6], kg/cow/day	32.32	33.55	33.26	1.82	0.87
Milk fat content, %	4.22	3.89	3.73	0.15	0.09
Milk fat production, kg/day	1.30	1.28	1.22	0.06	0.58
Fat corrected milk, kg/day	32.08	32.52	31.50	1.50	0.88
Protein content, %	3.47	3.39	3.38	0.07	0.67
Protein production, kg/day	1.08	1.11	1.11	0.05	0.82
Lactose content, %	4.99	4.98	5.02	0.03	0.60
Lactose production, kg/day	1.58	1.64	1.69	0.09	0.70
SCC[7], $\times 10^{-3}$ CFU/ml	198	346	205	87.40	0.41
Urea content, mg/dl	23.20	21.20	21.60	0.91	0.27

[1] AURA – unextracted *Aurantiochytrium limacinum* containing 17.04 g DHA/100 g (Alltech Inc., Nicholasville, KY, USA), so APC and AMC groups were supplemented with 20.1 and 25.1 g of DHA/cow/day, respectively; [2] twelve cows from each treatment group were sampled on four occasions (day 0, 28, 56 and 84) giving 48 milk samples per treatment group overall, with the data presented analysed as repeated measures; [3] body condition score on day 84: 1 – emaciated; 2 – thin; 3 – moderate; 4 – stout; 5 – obese; [4] mean of day 7–84, data analysed using an ANOVA with repeated measures; [5] daily TMR intake (dry matter (DM) basis) per cow estimated from total TMR intake per pen/ number of cows per pen; [6] mean of day 1–84 data analysed using an ANOVA with repeated measures; [7] SCC – somatic cell counts; [8] S.E.M. – standard error of the mean

merically higher count was observed for the APC group due to consistently high values detected in the milk of a single cow at every time point (1511, 1253, 1369 and 1650 $\times 10^3$ CFU/ml on days 0, 28, 56 and 84, respectively). Near the end of the study a numerical increase in milk yield was observed for the treatment groups with the APC and AMC groups yielding up to 1.68 kg and 2.27 kg, respectively, more milk than the CPC group. The fat content (%) of the milk differed between the groups on day 56 with the AMC group having a significantly lower fat content than the CPC group (Table 4; $P = 0.038$). A similar trend was evident in the repeated measures analysis for the entire study period in which the AMC group tended towards a lower fat content (%) than the control ($P = 0.087$). No differences in FCM, protein, lactose or urea content were observed at any time point or over the course of the whole experiment.

Milk fatty acid profile

No DHA was detected in the milk of any of the groups at the beginning of the study and no DHA was detected in the milk of cows from the control group at any time point during the experiment. On each sampling day the AMC group has significantly more DHA (g/100 g fatty acids) than the APC and the CPC groups (Table 4). A similar pattern of enrichment over time was observed for both groups (Figure 2). Overall, the repeated measures analysis indicated that the mean DHA content of milk from the APC group was significantly higher than the control, while that from AM group was significantly higher than both other groups ($P < 0.001$). The level of EPA detected in the milk differed on day 28, on

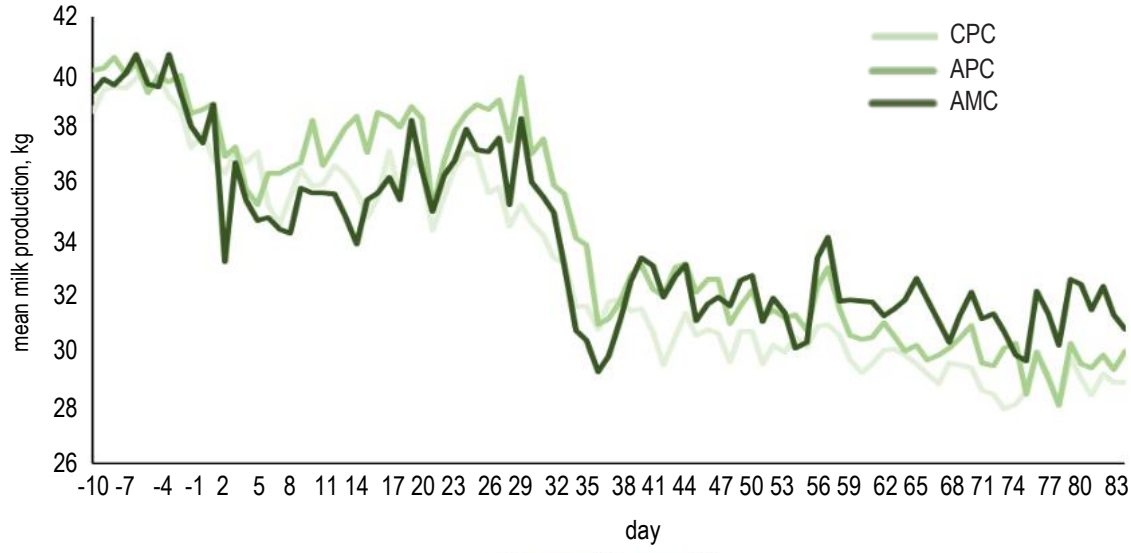

Figure 1. Milk yield (kg/day) from cows fed a control pellet (CPC) or docosahexaenoic acid (DHA)-rich microalgae *Aurantiochytrium limacinum* supplemented diet in either pellet (APC) or meal (AMC) form for an 84-day treatment period with a 10-day pre-treatment

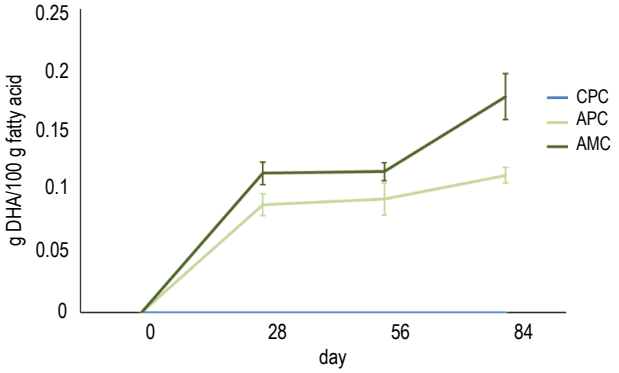

Figure 2. Milk docosahexaenoic acid (DHA) concentration (g/100 g fatty acids ± 95% C.I.) from cows fed a control diet (CPC) or diets supplemented with DHA-rich microalgae in pellet (APC) or meal (AMC) form

which the milk from the APC and AMC groups had significantly more EPA in the milk fat than the control group ($P = 0.001$), while on day 84 the AMC group has significantly more EPA than both other groups (Table 4). The n-6:n-3 ratio was the lowest for the AMC group, the difference being significant between AMC and both CPC and APC. The n-6:n-3 ratio was significantly lower for APC than for CPC ($P < 0.0001$ in each case). The results of the repeated measures analysis for the overall FA profiles of the experimental groups are shown in Table 5.

As TMR intake was recorded per pen and not per cow, the actual DHA intake of each animal could not be determined. The individual DHA intake was estimated based in the intake for the pen divided by the number of cows in the pen. Dietary supplementation for a period of 84 days with 20.5 and 25.1 g DHA/cow/day resulted in 4.47 and 6.37 mg DHA/100 ml milk, equating to 44.7 and 63.7 mg/l of milk, respectively for the APC and AMC groups. The estimated individual intake and mean concentration of DHA detected in the milk were used to calculate the efficiency of transfer of DHA from the feed to the milk using the following formula: DHA in milk (mg/d) / DHA intake (mg/d) × 100. The transfer efficiencies calculated for each sampling day and overall are shown in Table 6.

Milk cheese making qualities

There were no differences between the control and treatment groups in terms of their milk casein content, milk fat natural creaming, or milk rennet coagulation properties at any time point or for the entire study period (Table 7). The difference between the groups in terms of milk titratable acidity showed tendency on day 28 ($P = 0.1$) with the AMC group having a lower value (2.53° SH/50 ml) than the CPC and APC groups (2.84 and 2.80° SH/50 ml, respectively).

Table 4. Milk production and composition parameters recorded for cows fed control pellet (CPC) or *Aurantiochytrium limacinum* (AURA[1]) supplemented pellet (APC) or meal (AMC) at four time points over the course of the study[2]

Indices	Control pellet	AURA pellet	AURA meal	S.E.M.[3]	*P*-value
Milk production, kg/cow/day					
days 10–0	39.08	39.72	39.43	2.291	0.980
days 1–28	36.07	37.53	35.89	2.081	0.831
days 29–56	31.34	33.02	32.36	1.878	0.818
days 57–84	29.26	30.09	31.53	1.696	0.638
Milk fat, %					
day 0	3.92	3.96	3.96	0.157	0.980
day 28	4.09	3.69	3.67	0.176	0.185
day 56	4.15^{b}	3.86^{ab}	3.54^{a}	0.161	0.038
day 84	4.42	4.14	3.98	0.171	0.196
Milk fat production					
day 0	1.46	1.51	1.46	0.062	0.828
day 28	1.38	1.38	1.27	0.077	0.514
day 56	1.26	1.22	1.16	0.057	0.488
day 84	1.26	1.24	1.21	0.066	0.881
Milk protein content, %					
day 0	3.37	3.35	3.35	0.084	0.986
day 28	3.37	3.28	3.26	0.076	0.567
day 56	3.46	3.42	3.36	0.076	0.633
day 84	3.57	3.47	3.53	0.082	0.696
Milk protein production, kg/day					
day 0	1.26	1.28	1.24	0.050	0.874
day 28	1.15	1.22	1.14	0.056	0.538
day 56	1.06	1.09	1.11	0.051	0.768
day 84	1.02	1.03	1.07	0.045	0.696
EPA[4], % of total fatty acids					
day 0	0.03	0.03	0.03	0.002	0.788
day 28	0.029^{b}	0.035^{a}	0.034^{a}	0.001	0.001
day 56	0.03	0.03	0.03	0.001	0.086
day 84	0.031^{b}	0.034^{b}	0.042^{a}	0.002	0.001
DHA[5], % of total fatty acids					
day 0	0.00	0.00	0.00	-	-
day 28	0.00^{c}	0.09^{b}	0.11^{a}	0.004	<0.001
day 56	0.00^{c}	0.09^{b}	0.12^{a}	0.004	<0.001
day 84	0.00^{c}	0.11^{b}	0.18^{a}	0.006	<0.001
n-6:n-3 ratio					
day 0	3.94	3.99	4.02	0.063	0.705
day 28	4.30^{a}	3.95^{b}	3.67^{c}	0.071	<0.001
day 56	4.06^{a}	3.74^{b}	3.60^{c}	0.043	<0.001
day 84	4.26^{a}	3.64^{b}	3.38^{c}	0.049	<0.001

[1] AURA – unextracted *Aurantiochytrium limacinum* containing 17.04 g DHA/100 g (Alltech Inc., Nicholasville, KY, USA), so APC and AMC groups were supplemented with 20.1 g and 25.1 g of DHA/cow/day, respectively; [2] twelve cows from each treatment group were sampled on four occasions: day 0, 28, 56 and 84; [3] S.E.M. – standard error of the mean; [4] EPA – eicosapentaenoic acid; [5] DHA – docosahexaenoic acid; [abc] – means within a row with different superscripts are significantly different

Table 5. Repeated measures analysis of the milk fatty acid composition (g/100 g of fatty acids) for cows fed control pellet (CP) or *Aurantiochytrium limacinum* (AURA[1]) supplemented pellet (APC) or meal (AMC) for 84 days[2]

Fatty acid	Control pellet	AURA pellet	AURA meal	S.E.M[3]	*P*-value
Butyric acid (C4:0)	1.74	1.76	1.73	0.041	0.907
Caproic acid (C6:0)	1.69	1.64	1.65	0.044	0.716
Caprylic acid (C8:0)	1.18	1.14	1.17	0.036	0.633
Capric acid (C10:0)	2.84	2.71	2.83	0.107	0.651
Undecanoic acid (C11:0)	0.07	0.06	0.05	0.006	0.173
Lauric acid (C12:0)	3.42	3.23	3.43	0.130	0.493
Tridecanoic acid (C13:0)	0.11	0.10	0.10	0.004	0.321
Myristic acid (C14:0)	11.94	11.74	12.19	0.242	0.431
Myristoleic acid (C14:1)	1.12^{ab}	1.06^{b}	1.29^{a}	0.060	0.031
Pentadecanoic acid (C15:0)	1.06	1.05	1.03	0.027	0.677
Palmitic acid (C16:0)	37.44	38.02	37.22	0.666	0.679
Palmitoleic acid (C16:1)	1.97	2.14	2.23	0.083	0.102
Heptadecanoic acid (C17:0)	0.47	0.47	0.46	0.010	0.831
cis-10-heptadecenoic acid (C17:1)	0.04	0.04	0.04	0.002	0.290
Stearic acid (C18:0)	9.99^{a}	8.87^{b}	8.55^{b}	0.307	0.006
Vaccenic acid (C18:1n9*trans*)	1.77^{b}	2.75^{a}	2.85^{a}	0.192	0.005
Oleic acid (C18:1n9*cis*)	20.00	19.56	19.41	0.599	0.771
C18:1n11*cis*	0.28^{b}	0.35^{a}	0.35^{ab}	0.019	0.033
Linoleic acid (C18:2n6*cis*)	1.71	1.82	1.89	0.058	0.103
Rumenic acid (C18:2)	0.39^{b}	0.64^{a}	0.67^{a}	0.037	<0.001
α-Linolenic acid (C18:3n3)	0.26	0.27	0.28	0.010	0.344
γ-Linolenic acid (C18:3n6)	0.04	0.03	0.03	0.001	0.074
Arachidic acid (C20:0)	0.11	0.12	0.11	0.003	0.589
cis-11,14,17-Eicosatrienoic acid (C20:3n3)	0.15^{a}	0.11^{b}	0.11^{b}	0.004	<0.001
cis-8,11,14-Eicosatrienoic acid (C20:3n6)	0.11^{a}	0.08^{b}	0.08^{b}	0.004	<0.001
Eicosapentaenoic acid (C20:5n3) (EPA)	0.03^{b}	0.03^{a}	0.04^{a}	0.001	0.001
Behenic acid (C22:0)	0.03^{b}	0.06^{a}	0.06^{a}	0.002	0.003
Docosahexaenoic acid (C22:6n3) (DHA)	0.00^{c}	0.10^{b}	0.14^{a}	0.004	<0.001
Lignoceric acid (C24:0)	0.03	0.03	0.03	0.001	0.002
Σ Short-chain fatty acids[4]	4.62	4.53	4.54	0.111	0.850
Σ Medium-chain fatty acids[4]	20.56	19.96	20.91	0.465	0.354
Σ Long-chain fatty acids[4]	74.83	75.51	74.54	0.520	0.414
Σ Saturated fatty acid	72.13	71.00	70.60	0.706	0.297
Σ Unsaturated fatty acid	27.87	29.00	29.40	0.706	0.297
Σ Monounsaturated fatty acid	25.17	25.91	26.16	0.648	0.540
Σ Polyunsaturated fatty acid (PUFA)	2.70^{b}	3.09^{a}	3.24^{a}	0.095	0.001
n-3 PUFA[5]	0.44^{c}	0.51^{b}	0.57^{a}	0.014	<0.001
n-6 PUFA[6]	1.87	1.94	2.01	0.059	0.247
n-6:n-3	4.21^{a}	3.78^{b}	3.55^{c}	0.046	<0.001

[1] AURA – unextracted *Aurantiochytrium limacinum* containing 17.04 g DHA/ 100 g (Alltech Inc., Nicholasville, KY, USA), so APC and AMC groups were supplemented with 20.1 and 25.1 g of DHA/cow/day, respectively; [2] twelve cows from each treatment group were sampled on four occasions (day 0, 28, 56 and 84) giving 48 milk samples per treatment group overall, with the data presented analysed as repeated measures; [3] S.E.M. – standard error of the mean; [4] the complete fatty acid profile included: short-chain fatty acids (from C4:0 to C8:0), medium-chain fatty acids (from C10:0 to C15:1) and long-chain fatty acids (from C16:0 to C22:6n3); [5] the total n-3 PUFA composition was calculated as the ∑ [α-linolenic acid (C18:3n3) + *cis*-11,14,17-eicosatrienoic acid (C20:3n3) + EPA (C20:5n3) + DHA (C22:6n3)]; [6] the total n-6 PUFA composition was calculated as the ∑ [linolelaidic acid (C18:2n6*trans*) + linoleic acid (C18:2n6*cis*) + γ-linolenic acid (C18:3n6) + *cis*-8,11,14-eicosatrienoic acid (C20:3n6) + arachidonic acid (C20:4n6)]; [abc] – means within a row with different superscripts are significantly different

Table 6. Estimated transfer efficiency (%) of docosahexaenoic acid (DHA) to milk from feeding either an *Aurantiochytrium limacinum* (AURA[1]) supplemented pellet (APC) or meal (AMC) to dairy cows as part of their total mixed ration (TMR) for an 84-day trial[2]

Group	Day	DHA in milk[1], g/100 g fatty acids	Milk production[1], kg/day	Milk fat[1], 100 g/day	Mean DHA in milk[3], mg/d	Mean DHA in milk[4], mg/100 ml	Estimated TMR intake[5], kg/cow/day	TMR DHA content[6], mg/kg	Estimated DHA intake[7], mg/cow/day	Estimated transfer efficiency[8],%
AURA pellet	28	0.089	37.53	13.8	1143	3.05	44.58	470	20953	5.46
	56	0.094	33.02	12.2	1065	3.23	44.94	450	19841	5.47
	84	0.113	29.26	12.4	1308	4.47	43.29	470	20229	6.47
	7–84	0.099	32.32	12.8	1176	3.64	43.55	470	20497	5.74
AURA meal	28	0.115	35.89	12.7	1359	3.79	43.04	580	26065	5.21
	56	0.116	32.36	11.6	1257	3.88	43.74	560	24388	5.15
	84	0.178	31.35	12.1	2007	6.37	43.61	570	24392	8.05
	7–84	0.136	32.36	12.2	1550	4.81	44.06	570	25114	6.17

[1] AURA – unextracted *Aurantiochytrium limacinum* containing 17.04 g DHA/100 g (Alltech Inc., Nicholasville, KY, USA), so APC and AMC groups were supplemented with 20.1 and 25.1 g of DHA/cow/day, respectively; [2] twelve cows from each treatment group were sampled on four occasions (day 0, 28, 56 and 84) giving 48 milk samples per treatment group overall; [3] mean DHA content in milk (mg/day) was calculated using the following formula: (0.933 × milk fat (100 g/day) × DHA (mg/100 g fatty acid) × 1000 (Glasser et al., 2007) in fact, the proportion of fatty acyl radicals (i.e., FA from which the OH group has been removed; [4] mean DHA in milk (mg/100 ml) calculated as (mean DHA in milk (mg/day) / milk production (kg/day)) / 10; [5] TMR intake (as fed) recorded per pen and divided by the number of animals per pen to achieve an estimation of mean intake per cow; [6] DHA concentration of the TMR for the preceding 28-day period; [7] estimated TMR content = estimated TMR intake (kg/cow/day) × TMR DHA content (mg/kg); [8] estimated transfer efficiency (%) of DHA from diet to milk = DHA in milk (mg/day) / DHA intake (mg/day) × 100

Table 7. Cheese making qualities of milk from cows fed control pellet (CPC) or *Aurantiochytrium limacinum* (AURA[1]) supplemented pellet (APC) or meal (AMC) for the duration of 84 days[2]

Days from the start of the study	Control pellet	AURA pellet	AURA meal	S.E.M.[3]	Treatment effect *P*-value	Days from the start of the study	Control pellet	AURA pellet	AURA meal	S.E.M.[3]	Treatment effect *P*-value
Milk casein, %						Milk rennet: clotting time (r30)					
day 0	2.61	2.58	2.61	0.06	0.90	day 0	22.23	20.36	19.40	1.70*	0.50
day 28	2.53	2.44	2.45	0.05	0.41	day 28	22.74	21.03	22.20	1.88*	0.80
day 56	2.61	2.54	2.57	0.05*	0.60	day 56	22.19	24.41	25.39	1.97	0.51
day 84	2.74	2.65	2.68	0.06	0.60	day 84	22.24	24.52	25.16	1.81*	0.53
days 7–84[4]	2.63	2.54	2.57	0.05*	0.40	days 7–84[4]	22.39	23.32	24.25	1.17*	0.50
Milk titratable acidity, SH/50 ml						Milk rennet: curd firming time (k20)					
day 0	3.44	3.37	3.22	0.11	0.40	day 0	7.10	7.74	8.07	0.63*	0.55
day 28	2.84	2.80	2.53	0.11	0.10	day 28	7.80	7.13	8.26	0.77*	0.59
day 56	3.16	3.07	3.04	0.14*	0.82	day 56	7.24	8.19	8.47	0.95	0.63
day 84	3.13	2.99	2.98	0.12	0.57	day 84	7.15	7.34	8.45	0.73*	0.42
days 7–84[4]	3.15	3.06	2.95	0.11*	0.42	days 7–84[4]	7.36	7.58	8.39	0.55*	0.40
Milk fat natural creaming, %						Milk rennet: curd firmness (a30)					
day 0	48.29	49.69	51.61	2.59	0.66	day 0	19.48	21.39	20.59	3.22*	0.91
day 28	55.72	62.65	56.82	2.82	0.19	day 28	15.44	18.07	17.22	2.46*	0.74
day 56	55.56	57.09	49.64	3.11*	0.20	day 56	16.62	13.38	10.13	2.57	0.23
day 84	57.92	58.00	55.00	3.22	0.76	day 84	20.67	15.11	14.39	3.35*	0.40
days 7–84[4]	56.23	59.26	53.80	2.37*	0.28	days 7–84[4]	17.72	15.53	13.80	1.53*	0.35

[1] AURA – unextracted *Aurantiochytrium limacinum* containing 17.04 g DHA/100 g (Alltech Inc., Nicholasville, KY, USA), so APC and AMC groups were supplemented with 20.1 and 25.1 g of DHA/cow/day, respectively; [2] twelve cows from each treatment group were sampled on four occasions (day 0, 28, 56 and 84) giving 48 milk samples per treatment group overall; [3] S.E.M. – standard error of the mean; [4] data analysed as repeated measures; * harmonic means

Discussion

The fatty acid composition of the milk from cows fed diets supplemented with a DHA-rich thraustochytrid *Aurantiochytrium limacinum* biomass (AURA), provided in either a pelleted or meal form, was successfully altered and improved to contain significantly higher levels of DHA than the control group, in addition to improved n-6:n-3 ratio. This finding is in agreement with similar recent trials

investigating the effect of supplementation with *A. limacinum* on milk fatty acid content (Moran et al., 2017b; 2018b). Both studies demonstrated a significant increase in the DHA content of milk following supplementation with AURA, with milk DHA concentrations increasing rapidly during the first weeks of supplementation until reaching their initial peaks at day 56, after which a similar level of enrichment was maintained in both studies. In the current study, a plateau between day 28 and 56 was observed for both groups, followed by a final increase to reach peak DHA concentrations by day 84. This pattern of enrichment is in contrast to the previous two studies. In the current study, on day 28, the ambient temperature rose from approximately 22 °C to 30 °C. After this point a decrease in milk production (Figure 1) and DM intake (results not presented) was observed for all cows on the trial in the days following day 28. Decreased dry matter intake and milk production are associated with heat-stress in dairy cows (Knapp and Grummer, 1991; West, 2003). The differing patterns of enrichment between this and the previous studies are likely due to the heat-stress experienced by the animals during the period directly after day 28.

Stability to pelleting was measured by recovery of DHA (%) from the pellet concentrate and subsequent TMR and from the meal version of the concentrate and subsequent TMR. Less DHA was recovered in the TMR of the APC diet than the AMC diet (4.7 vs 5.7 g/kg, respectively). The target level of DHA intake was 25.5 g per cow per day. The estimated DHA intake was found to range between 19–21 g/day and 24–26 g/day for the APC and AMC diets, respectively, with only the latter meeting the expected DHA intake level. Therefore, the pelleting conditions used in this study appear to decrease the quantity of DHA recovered in the pellet concentrate. The pellet concentrate was produced from the meal concentrate so it is unlikely to be a mixing error. The lower DHA content of the APC feed was reflected in the DHA concentrations of the milk, with the APC group having a significantly lower DHA content than the AMC group on days 28, 84 and for the study overall.

The transfer efficiencies calculated based on the estimated individual intake of DHA ranged between 5 and 8%. As the DHA intake was not recorded on an individual basis, the difference in transfer efficiencies between the APC and AMC groups could not be compared statistically. Other studies have previously reported a broad range of DHA transfer efficiencies from feed to milk, 1–21.6%, and the efficiencies observed in this study fall within this range (Franklin et al., 1999; Chilliard et al., 2001; Boeckaert et al., 2008; Stamey et al., 2012; Moate et al., 2013; Moran et al., 2017b). The authors found no other study investigating the influence of pelleting on DHA stability in a dairy concentrate feed. However, in a previous study that investigated the effect of pelleting diets for laying hens, containing an AURA biomass, on the recovery of DHA from pellets and concomitant DHA transfer from feed to the egg yolk, the process of pelleting did not reduce the DHA content of the feed and transfer to the egg yolk was similar for birds fed pelleted or meal diets (Moran et al., 2017a). DHA, with numerous methylene-interrupted ethylenic double bonds, is sensitive to temperature with non-volatile degradation products formed, including polymers, cyclic fatty acid monomers and geometrical isomers of DHA formed above 180 °C (Fournier et al., 2006). This temperature is significantly higher than that used in this study. Therefore, further work needs to be performed on the stability and recovery of DHA in complex feed matrices particularly when provided as marine protist ingredient.

Supplementation with AURA resulted in similar milk yields for control and treatment groups. Approaching the end of the study, however, the milk yield for the supplemented groups was numerically higher. This trend was observed in a similar study following supplementation with AURA over an 84 day period, with the greatest differences in milk yield observed between days 50 and 84 (Moran et al., 2017b). Previous studies have reported a potential negative impact of algae supplementation (Boeckaert et al., 2008), as well as indicated no impact on ruminant milk yield (Bichi et al., 2013; Moate et al., 2013). The duration of supplementation, the stage of lactation, background diet, and the quantity of algae provided are likely responsible for these differences (Moran et al., 2017b).

Overall, supplementation had no effect on the fat content (%) of the milk, however, on day 56 the AMC group had a significantly lower fat content than the CPC group (3.54 vs 4.15; $P = 0.04$). A trend towards milk fat depression was observed for the study overall for the AMC group (3.73 vs 4.22; $P = 0.087$). Milk fat depression as a result of supplementation with DHA-rich ingredients has been previously reported and occurs as a result of the inhibition of *de novo* milk FA production (Papadopoulos et al., 2002; Boeckaert et al., 2008; Moate et al., 2013; Moran et al., 2017b). The DHA intake was found to be 20.4 and 25.1 g DHA/day for the APC and AMC groups,

respectively, and only the AMC group with the higher DHA intake displayed a trend towards milk fat depression. In contrast, in a similar study, a DHA intake of 22.9 g DHA/day resulted in significant depression of milk fat (Moran et al., 2017b). It is likely that the discrepancies between these studies can be attributed to the heat-stress experienced by the cows in the current study, in addition to differences in the background diet of the cows in each study. The FCM however, was not affected by supplementation, which is in agreement with previous studies (Papadopoulos et al., 2002; Moran et al., 2017b; 2018b). In addition, milk protein, lactose and urea content were not affected by supplementation and were in agreement with the findings of other authors (Stamey et al., 2012; Moate et al., 2013; Moran et al., 2017b; 2018b).

The FA profile of cheese has been shown to be similar to the FA profile of the milk used to make it (Allred et al., 2006; Nudda et al., 2014; Bodkowski et al., 2016). Bodkowski et al. (2016) supplemented animal feed with synthesised CLA and n-3 rich fish oil, successfully enriching the milk with 0.16 and 0.14 g DHA/100 g FA after 14 and 30 days of the supplementation, respectively. The corresponding cheeses were found to have 0.09 and 0.14 g DHA/100 g FA maintaining similar levels of DHA as the milk used for cheese production. However, the use of PUFA enriched milk may have an impact on the cheese making properties. Avramis et al. (2003) demonstrated that milk from cows fed diets supplemented with fish meal differ in casein micelle size, protein distribution and fat globule diameter, differences that were likely to result in altered processing properties. In this study, none of the cheese making properties investigated differed significantly between the control and treatment groups. No significant differences were observed between the control and treatment groups in terms of clotting time, curd firming time and curd firmness, however further work is required to determine the effect of a greater level of supplementation on these cheese making properties. Depending on the type of cheese being produced, changes to the cheese making properties can be beneficial with faster ripening time and a more desirable texture reported for cheddar cheese produced from the milk of cows fed diets supplemented with fish meal (Avramis et al., 2003).

In the European Union, to make the nutritional claim that a food is a source of n-3 PUFA, it must contain at least 40 mg of EPA + DHA per 100 g fresh weight and per 100 kcal. As such, the enriched milk from the current study would not be considered a source of n-3 PUFA. In 2018, the average price paid to milk producers in the EU was approximately €0.36 (European Commision, 2018). Feeding supplementary AURA (€7.00/kg) at the same rate of the current study (150 g/day) would cost approximately €1.05 per cow per day. In our study the cost of supplementing both the APC and AMC group would be €0.03 per l of milk produced. Considering a nutritional claim to increase the value of the milk, it would not make financial sense to incorporate AURA in production of fresh milk. In addition, in cases where n-3 PUFA enriched milk is sold, it provides consumers with the lowest value for money when compared with other n-3 PUFA enriched foods or supplements (Watters et al., 2012). Using DHA enriched milk to produce cheese however, could be a more desirable option for dairy processors. Based on the Van Slyke yield equation (Mullan, 2008) the theoretical yield of cheese from the milk of the AP group can be calculated as follows:

$$\text{cheese yield} = \frac{[(\%\ \text{milk fat} \times 0.93) + (\%\ \text{milk casein} \times 0.96)] \times 109}{100 - \%\ \text{cheese moisture}}$$

if we use: % milk fat – 3.89, % milk casein – 2.54 and % cheese moisture –35, 1 l of milk would produce approximately 102 g of cheddar cheese with a similar cheese yield observed for both the CPC and AMC groups. As the fatty acid profile of cheese is similar to the milk used to produce it (Allred et al., 2006), both the cheese of the APC and AMC groups could be expected to contain 44.7 and 63.7 mg of DHA/100 g respectively, meeting the criteria to be considered a source of n-3 PUFA, and allowing dairy farmers and processors to charge a premium for these enriched products.

Conclusions

Dietary supplementation with a docosahexaenoic acid (DHA)-rich thraustochytrid *Aurantiochytrium limacinum* biomass (AURA) at a dose of 150 g/cow/day (which gives about 24 g DHA/cow/day) for 84 days resulted in the successful enrichment of milk with DHA and a nutritionally preferred decreased n-6:n-3 ratio. However the process of pelleting resulted in a lower content of DHA in feed, so better results for DHA milk content were stated for AURA supplemented in meal form. The examined level of supplementation had no effect on other various milk properties (fat, protein, lactose content) and its production yield. In addition, with no differences observed between the supplemented and control animals in terms of cheese making properties, the milk from each group would be

similarly suitable for cheese production at this level of enrichment. However future work should investigate the influence of the DHA-rich milk obtained by AURA addition into cow diet on the maturation time and flavour profile of the cheese.

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