Recombinant lactobacilli expressing linoleic acid isomerase can modulate the fatty acid composition of host adipose tissue in mice

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We have previously demonstrated that oral administration of a metabolically active Bifidobacterium breve strain, with ability to form cis-9, trans-11 conjugated linoleic acid (CLA), resulted in modulation of the fatty acid composition of the host, including significantly elevated concentrations of c9, r11 CLA and omega-3 (n-3) fatty acids in liver and adipose tissue. In this study, we investigated whether a recombinant lactobacillus expressing linoleic acid isomerase (responsible for production of t10, c12 CLA) from Propionibacterium acnes (PAI) could influence the fatty acid composition of different tissues in a mouse model. Linoleic-acid-supplemented diets (2 %, w/w) were fed in combination with either a recombinant t10, c12 CLA-producing Lactobacillus paracasei NFBC 338 (Lb338), or an isogenic (vector-containing) control strain, to BALB/c mice for 8 weeks. A third group of mice received linoleic acid alone (2 %, w/w). Tissue fatty acid composition was assessed by GLC at the end of the trial. Ingestion of the strain expressing linoleic acid isomerase was associated with a 4-fold increase (P<0.001) in t10, c12 CLA in adipose tissues of the mice when compared with mice that received the isogenic non-CLA-producing strain. The livers of the mice that received the recombinant CLA-producing Lb338 also contained a 2.5-fold (albeit not significantly) higher concentration of t10, c12 CLA, compared to the control group. These data demonstrate that a single gene (encoding linoleic acid isomerase) expressed in an intestinal microbe can influence the fatty acid composition of host fat.

INTRODUCTION

Evidence is emerging to support the concept that the enteric microbiota can exert profound effects on human health and disease, involving complex host–bacteria interactions that are as yet poorly understood. The gut microbiota is important to the host with regard to metabolic functions, providing nutrients and conferring an ability to resist bacterial infections (Marchesi & Shanahan, 2007; Wilks, 2007), as well as playing a dominant role in the education of the intestinal mucosal immune responses. Furthermore, the enteric microbiota has been shown to exert effects on disease processes outside the gut, including an impact on obesity and non-alcoholic fatty liver disease (Dumas et al., 2006; Marchesi & Shanahan, 2007).

Conjugated linoleic acid (CLA) is a beneficial bacterial metabolite formed via microbial isomerization of linoleic acid. CLA is a collective term describing different isomers of linoleic acid with conjugated double bonds, the cis-9, trans-11 (c9, t11) CLA isomer being the most abundant form and the t10, c12 CLA isomer accounting for ~1 % of total milk fat CLA (Jensen, 2002). It has been reported that t10, c12 CLA is the most potent isomer in terms of potential to prevent cell proliferation and induce apoptosis in cancer cells (Cho et al., 2005, 2006; Kim et al., 2002a; Lee et al., 2006b; Ochoa et al., 2004). t10, c12 CLA is also associated with decreased body fat and increased lean body mass in various animal species, such as mice (Park et al., 1997), chickens (Cherian et al., 2005), rats (Yamasaki et al., 2003), hamsters (Navarro et al., 2006), pigs (Ostrowska et al., 1999) and, to some extent, humans (Blankson et al., 2000; Mougiou et al., 2001; Risérus et al., 2001; Smedman & Vessby, 2001; Thom et al., 2001). Commensal Bifidobacterium and Lactobacillus species from the mammalian gastrointestinal
tract have been shown to produce CLA in vitro (Barrett et al., 2007; Coakley et al., 2003; Rosberg-Cody et al., 2004). The majority of these studies have demonstrated production of c9, t11 CLA from linoleic acid, while only a few bacterial species have been reported to produce t10, c12 CLA, i.e. Propionibacterium acnes isolated from mouse caecum (Verhulst et al., 1997), the rumen bacterium Meganthera elsdonii (Kim et al., 2002b) and the human-derived Lactobacillus rhamnosus PL60 and Lactobacillus planatarum PL62 (Lee et al., 2006a; Lee et al., 2007). However, only a few studies have established CLA production in vivo following ingestion of a CLA-producing bacterium. For example, the in vivo production of t10, c12 CLA by Lactobacillus rhamnosus PL60 and Lactobacillus planatarum PL62 was reported (Lee et al., 2006a, 2007), and we recently demonstrated that feeding different animal species a c9, t11 CLA-producing Bifidobacterium strain of human origin (Bifidobacterium breve NCIMB 702258), in combination with dietary linoleic acid, resulted in modulation of the fatty acid composition of the host, including significantly elevated concentrations of c9, t11 CLA in the liver (Wall et al., 2009).

The enzyme Propionibacterium acnes isomerase (PAI) is responsible for conversion of linoleic acid to the t10, c12 CLA isomer and has previously been expressed in bacteria, yeast and tobacco seeds (Horneung et al., 2005). We have successfully cloned and overexpressed PAI in Lactococcus lactis, resulting in conversion rates of >50% of linoleic acid to t10, c12 CLA (Rosberg-Cody et al., 2007). Moreover, when the microbially derived t10, c12 CLA was incubated with SW480 colon cancer cells for 5 days, cell viability was decreased by 92% (Rosberg-Cody et al., 2007). The objective of this study was to investigate whether a recombinant Lactobacillus paracasei NFBC 338 (Lb338), expressing the gene encoding PAI, would result in elevated tissue concentrations of t10, c12 CLA in vivo.

**METHODS**

**Cultures and media.** The probiotic strain Lactobacillus paracasei NFBC 338 (Lb338) was previously isolated from the human gastrointestinal tract, and obtained from University College Cork, Ireland, under a restricted materials transfer agreement. Lb338 was routinely cultured overnight (~17 h) in de Man–Rogosa–Sharpe (MRS) broth (Oxoid, England) and incubated at 37°C under anaerobic conditions in anaerobic jars containing AnaeroCult A gas packs (Merck). Lb338 harbouring the vector pMSP3535 was routinely cultured with erythromycin (10 μg ml⁻¹; Sigma) as a selective marker.

**DNA manipulation.** The 1278 bp coPAI gene (codon-optimized Propionibacterium acnes isomerase in a plant vector; BASF) encoding the linoleic acid isomerase from P. acnes (Horneung et al., 2005), was amplified and cloned into the nisin-inducible lactococcal plasmid pNZ8048 (containing the nisA promoter) as described previously (Rosberg-Cody et al., 2007). The coPAI gene was excised from pNZ8048-coPAI using PstI and XbaI restriction enzymes and ligated into the same sites of the Lactobacillus nisin-inducible plasmid pMSP3535 (Bryan et al., 2000), as described by the supplier (New England Biolabs), resulting in the construct pEcoPAI (Fig. 1). The recombinant plasmid was double-digested with the same enzymes to verify the correct clone and subsequently electroporated into Lb338. Electroporant Lb338 cells were prepared using 3.5 × SMEB (1 M sucrose, 3.5 mM MgCl₂), as described by Luchansky et al. (1988). Sequence analysis was performed using DNASTAR software. The Qiagen Plasmid Mini kit was used to isolate plasmid DNA from Lb338 with one minor modification: 40 mg lysozyme ml⁻¹ was added to buffer P1 and incubated for 2 h at 37°C. PCR products were purified using a Qiaquick PCR Purification kit (Qiagen).

**In vitro conversion of t10, c12 CLA by recombinant lactobacilli.** The recombinant Lb338 was assessed for its ability to convert free linoleic acid (0.5 mg ml⁻¹; Sigma) to t10, c12 CLA as follows. A 1% (v/v) inoculum of an overnight culture was transferred to 10 ml broth (Lb338) as a selective marker. The standard diet had the following components: 9613 bp

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**Fig. 1.** Plasmid construct pEcoPAIL used to transform Lb. paracasei NFBC 338 (Lb338). ‘coPAI’ denotes the codon-optimized P. acnes isomerase gene (t10, c12 CLA isomerase).
nutrient composition: nitrogen-free extract (57.39 %), crude protein (18.35 %), moisture (10 %), ash (6.27 %), crude fibre (4.23 %) and crude oil (3.36 %), which consisted of saturated fatty acids [lauric acid (C12:0, 0.03 %), myristic acid (C14:0, 0.14 %), palmitic acid (C16:0, 0.33 %) and stearic acid (C18:0, 0.06 %)], monounsaturated fatty acids [myristoleic acid (C14:1, 0.02 %), palmitoleic acid (C16:1, 10 %) and oleic acid (C18:1, 0.87 %)] and polyunsaturated fatty acids [linoleic acid (C18:2n-6, 0.96 %), linolenic acid (C18:3n-3, 0.11 %) and arachidonic acid (C20:4n-6, 0.11 %)].

For linoleic acid treatment (added as trilinolein, with linoleic acid being the only fatty acid; Larodan Fine Chemicals), a powdered diet [milled standard non-purified CRM(P) pellets blended with the fatty acid was administered to each animal daily to yield approximately 180 mg linoleic acid per day. The mice would consume this diet within a few minutes. Mice were individually caged and exposed to a 12 h light/dark cycle, maintained at a constant temperature of 25 °C.

All laboratory animal experiments were performed according to the guidelines for the care and use of laboratory animals approved by the Department of Health and Children of the Irish Government. One week after arrival, mice were divided into three groups for different dietary treatments. Group A (n=8) received a linoleic-acid-supplemented diet (2 %, w/w, Larodan Fine Chemicals) and approximately 1 × 10⁸ live r0, c12 CLA-producing Lb338 (carrying the plasmid construct pEcoPAIL) per day. The probiotic culture was administered by resuspending appropriate quantities of freeze-dried powder in the water that the mice consumed. Group B (n=8) received the linoleic-acid-supplemented diet (2 %, w/w) in combination with approximately 1 × 10⁸ live non-CLA-producing Lb338 (carrying the empty plasmid pMSP3535) per day. Group C (n=5) received a linoleic-acid-supplemented diet (2 %, w/w) and placebo freeze-dried powder (15 %, w/v, trehalose in distilled H2O). After 8 weeks on the experimental diets, the mice were sacrificed by cervical dislocation, and tissues were removed, blotted dry on filter paper, weighed and frozen in liquid nitrogen. All samples were stored at −80 °C until processed.

Microbial analysis. Microbial analysis of faecal samples collected from all groups every 2 weeks involved enumeration of Lb338 by pour-plating serial dilutions onto MRS agar supplemented with erythromycin (10 μg ml⁻¹, Sigma). Agar plates were incubated anaerobically for 72 h at 37 °C. Anaerobic environments were created using CO₂-generating kits (Anaerocult A) in sealed gas jars.

Lipid extraction and fatty acid analysis. Lipids were extracted with chloroform/methanol (2:1, v/v) according to the method of Folch et al. (1957). Fatty acid methyl esters (FAMEs) were prepared using first 10 ml 0.5 M NaOH in methanol for 10 min at 90 °C followed by 10 ml 14 % BF₃ in methanol (Sigma) for 10 min at 90 °C (Park & Goins, 1994). FAMEs were recovered with hexane. Prior to GLC analysis, samples were dried over 0.5 g anhydrous sodium sulphate for 1 h and stored at −20 °C. FAMEs were separated by GLC (Varian 3400 instrument fitted with a flame-ionization detector) using a Chrompack CP Sil 88 column (100 m × 0.25 mm i.d., 0.20 μm film thickness) and helium as carrier gas. The column oven temperature was initially held at 80 °C for 8 min, and then increased at 0.5 °C min⁻¹ to a final temperature of 200 °C. The injection volume was 0.6 μl with automatic sample injection on a SPI 1093 splitless on-column temperature programmable injector. Data were recorded and analysed on a Minichrom PC system (VG Data Systems). Peaks were identified with reference to retention times of fatty acids in a standard mixture. The percentage of individual fatty acids was calculated according to the peak area relative to the total area (total fatty acids were set at 100 %). Results are shown as mean ± SEM per 100 g FAMEs.

Statistical analysis. Data are presented as means ± SEM per group of mice. Data were analysed using analysis of variance (ANOVA) followed by Tukey’s post-hoc test using GraphPad InStat version 3.05.

RESULTS

Microbial production of t10, c12 CLA by recombinant L. paracasei NFBC 338 (Lb338)

Lb338 harbouring the nisin-inducible Lactobacillus plasmid containing the c0PAI gene, pEcoPAIL (Fig. 1), converted approximately 30 % of linoleic acid to r10, c12 CLA, following induction at OD₆₀₀ 0.5 with 50 ng nisin ml⁻¹ and incubation for 48 h in the presence of the free fatty acid substrate (0.5 mg ml⁻¹). However, r10, c12 CLA production by uninduced cells of Lb338 pEcoPAIL was similar to that obtained with nisin-induced cells (24.4 ± 0.4 % and 28.9 ± 0.5 % conversion of substrate to product, respectively). Lb338 transformed with the empty vector pMSP3535 did not convert any of the added linoleic acid to r10, c12 CLA.

Survival and gastric transit of administered lactobacilli and confirmation of ability to produce t10, c12 CLA following gastric transit

Quantification of the numbers of bacteria of the recombinant CLA-producing Lb338 (group A) and the non-CLA-producing isogenic control Lb338 (group B) monitored in the faeces of mice confirmed gastrointestinal transit and survival of the administered strains. Stool recovery of the recombinant CLA-producing Lb338 and the non-CLA-producing isogenic control Lb338 was approximately 1 × 10⁹ c.f.u. (g faeces⁻¹) at week 2, which increased to approximately 1 × 10¹⁰ c.f.u. (g faeces⁻¹) at week 4 and remained at ~1 × 10⁹ c.f.u. (g faeces⁻¹) throughout the rest of the trial. Recovered Lactobacillus isolates (from groups A and B) were inoculated into MRS broth containing linoleic acid (0.5 mg ml⁻¹), which confirmed that the recovered Lb338 from group A, but not from group B, retained the ability to convert linoleic acid to r10, c12 CLA following passage through the gastrointestinal tract (data not shown). Oral administration of the recombinant CLA-producing Lb338 (group A) and the non-CLA-producing isogenic control Lb338 (group B) did not significantly influence body weight gain throughout the feeding trial (Fig. 2). In addition, liver weights were not significantly different between the groups (data not shown).

In vivo production of t10, c12 CLA by recombinant lactobacilli in mice

Following 8 weeks of dietary treatment, it was demonstrated that r10, c12 CLA was produced by the recombinant Lb338 in the gastrointestinal tract of mice. The mean t10, c12 CLA content of the adipose tissue was 4-fold higher in the mice that received the recombinant CLA-producing
Lb338 in combination with linoleic acid (group A) compared with the isogenic control (group B) (P, 0.001, Fig. 3). This higher concentration of t10, c12 CLA in group A coincided with an approximately 20% lower concentration of linoleic acid in the adipose tissue (although the difference was not statistically significant) in comparison to group B (Table 2). Oral administration of the recombinant CLA-producing Lb338 (group A) also resulted in a 2.5-fold higher concentration of t10, c12 CLA in the livers compared with group B (non-CLA-producing Lb338), although the difference was not statistically significant (Table 1).

**Dietary supplementation with probiotic Lb338 changes host tissue fatty acid composition**

Changes in tissue fatty acid composition caused by dietary supplementation with the probiotic Lb338 were investigated by comparing the groups of mice that received Lb338 (groups A and B) with the group that received linoleic acid alone (group C).

The mice that received Lb338 (groups A and B) had a significantly lower concentration of oleic acid in the spleen (P<0.01) and a numerically lower concentration of oleic acid in the adipose tissue compared to mice that received linoleic acid alone (Fig. 4). Furthermore, administration of Lb338 (groups A and B) resulted in lower concentrations of arachidonic acid in the liver (17% and 19%, lower, respectively) compared to mice that received linoleic acid alone (group C), although the differences were not statistically significant (Table 1).

**DISCUSSION**

This study demonstrates that t10, c12 CLA production occurred in vivo when a recombinant Lactobacillus strain harbouring the gene encoding linoleic acid isomerase was administered to mice. As a direct consequence of this, the t10, c12 CLA content of adipose tissue was elevated fourfold in animals that received the recombinant t10, c12 CLA-producing Lb338 in combination with linoleic acid compared to mice that received the isogenic control strain. t10, c12 CLA has been shown to be beneficial to host health (Kim et al., 2002a; Ochoa et al., 2004; Yamasaki et al., 2004).
2003) and has been demonstrated to alter body composition by reducing the fat content and increasing the lean body mass in animal studies, and also in some human studies (Blankson et al., 2000; Smedman & Vessby, 2001; Thom et al., 2001). It was reported by Lee et al. (2006a) that t10, c12 CLA was produced in vivo when the human-originated bacterium *L. rhamnosus* PL60 was fed to diet-induced obese mice. The relatively low amount of t10, c12 CLA produced by this strain (1.9 μg ml⁻¹ was detected in the sera) was enough to produce an anti-obesity effect. Moreover, liver steatosis, a well-known side effect of t10, c12 CLA, was not observed following *L. rhamnosus* PL60 treatment in this study (Lee et al., 2006a). Thus, the lower dose of CLA produced by a probiotic strain may be a reliable solution to control the adverse effects caused by dietary t10, c12 CLA, i.e. liver steatosis. In addition, we have previously shown that the microbially derived t10, c12 CLA, at a concentration of 20 μg ml⁻¹, has the ability to kill 92% of human SW480 colon cancer cells when co-incubated with these cells for 5 days (Rosberg-Cody et al., 2007). In contrast to dietary CLA, which is primarily absorbed from the small intestine, using a t10, c12 CLA-producing strain would result in the provision of t10, c12 CLA directly and continuously to the large intestinal lumen, where this fatty acid would exhibit anti-inflammatory and anti-proliferative effects on colonocytes (Bassaganya-Riera et al., 2004; Kemp et al., 2003), thereby contributing to colonic mucosal homeostasis.

Although the t10, c12 CLA content of adipose tissue was significantly higher in the animals that received the t10, c12 CLA-producing recombinant strain, lower concentrations of t10, c12 CLA was also detected in animals that did not receive this strain. One possible explanation for this may be that the natural enteric microbiota of mice can produce some t10, c12 CLA, using linoleic acid as substrate. In this respect, it is worth noting that the origin of the PAI gene used in this study to transform *Lb338* is *Propionibacterium acnes*, a strain that was originally isolated from mouse caecum (Verhulst et al., 1987).

Interestingly, administration of *L. paracasei* NFBC 338 (both the recombinant strain and the isogenic control) in combination with linoleic acid resulted in lower concentrations of arachidonic acid in the liver (17% and 19% lower, respectively) compared to mice that received linoleic acid alone. Since the eicosanoids derived from arachidonic acid, such as the 2-series prostaglandins and the 4-series leukotrienes, are in general regarded as being pro-inflammatory in nature (Bagga et al., 2003), decreased amounts of arachidonic acid would be considered beneficial, especially in chronic inflammatory settings that are of high prevalence in Western societies such as inflammatory bowel disease, cardiovascular disease and obesity, which are characterized by an excessive production of arachidonic-acid-derived eicosanoids (Calder, 2001; James et al., 2000; Jupp et al., 2007; Wallace, 2001).

Administration of *L. paracasei* NFBC 338 also resulted in

Table 1. Fatty acid composition (%) of livers from BALB/c mice

Data are means (±SEM) (group A and group B, n=8; group C, n=5). Different superscript letters within a row indicate significant difference at *P*<0.05 based on ANOVA followed by Tukey’s post-hoc test.

<table>
<thead>
<tr>
<th>Fatty acid*</th>
<th>Fatty acid composition (g per 100 g FAMEs)</th>
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<tbody>
<tr>
<td></td>
<td>Group A (Lb338 + linoleic acid)</td>
</tr>
<tr>
<td>14:0</td>
<td>1.7 (0.26)bc</td>
</tr>
<tr>
<td>16:0</td>
<td>17.82 (0.80)</td>
</tr>
<tr>
<td>16:1c9</td>
<td>0.8 (0.08)</td>
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<tr>
<td>18:0</td>
<td>12.63 (1.07)</td>
</tr>
<tr>
<td>18:1c9</td>
<td>5.96 (0.34)</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>30.89 (2.07)</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>1.02 (0.68)</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.69 (0.09)</td>
</tr>
<tr>
<td>t10, c12 CLA</td>
<td>0.010 (0.003)</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>0.41 (0.03)</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.69 (0.09)</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>12.48 (1.05)</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.04 (0.003)</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.20 (0.007)</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>4.91 (0.20)c</td>
</tr>
</tbody>
</table>

*14:0, myristic acid; 16:0, palmitic acid; 16:1c9, palmitoleic acid; 18:0, stearic acid; 18:1c9, oleic acid; 18:2n-6, linoleic acid; 18:3n-3, linolenic acid; 18:3n-6, γ-linolenic acid; 18:4n-3, stearidonic acid; 20:3n-6, dihomoy-γ-linolenic acid; 20:4n-6, arachidonic acid; 20:5n-3, eicosapentaenoic acid; 22:5n-3, docosapentaenoic acid; 22:6n-3 docosahexaenoic acid.
significantly lower concentrations of oleic acid in the spleen and adipose tissue compared to mice that received linoleic acid alone. Furthermore, our data indicate that it was not the linoleic acid isomerase gene, but the administered probiotic lactobacilli that was responsible for the decreased oleic acid observed in probiotic-supplemented animals compared with unsupplemented controls. Previously, Kalavathy et al. (2006) reported that administration of a mixture of *Lactobacillus* to broiler chickens significantly reduced the concentrations of oleic acid in liver and muscle. A reduced concentration of oleic acid has also been observed in the liver of rats fed a mixture of probiotics (Fukushima et al., 1999). This lower concentration of oleic acid could possibly be due to a reduction in the synthesis of the fatty acid or a reduced absorption from the intestine.

In conclusion, this is to our knowledge the first report to demonstrate that oral administration of a genetically modified r10, c12 CLA-producing strain (containing the single gene for r10, c12 CLA production) results in modulation of the fatty acid composition of the host, including significantly higher concentrations of r10, c12 CLA in adipose tissue, which is directly attributable to the presence of the linoleic acid isomerase gene.

### ACKNOWLEDGEMENTS

The support of BASF Plant Science GmbH is gratefully acknowledged, as is the technical assistance of Seamus Aherner, Lee Siggens, Grainne Hurley and Frances O’Brien. The authors are supported, in part, by Science Foundation Ireland, The European Union (Project QLK1-2002-02362), the Irish Ministry for Food and Agriculture, the Higher Education Authority and the Health Research Board of Ireland and the Irish Government under the National Development Plan 2000–2006.

### REFERENCES


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### Table 2. Fatty acid composition (%) of adipose tissue from BALB/c mice

<table>
<thead>
<tr>
<th>Fatty acid*</th>
<th>Fatty acid composition (g per 100 g FAMEs)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Group A (Lb338 + linoleic acid)</td>
</tr>
<tr>
<td>14:0</td>
<td>2.0 (0.2)</td>
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<tr>
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<td>16:1c9</td>
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<tr>
<td>18:0</td>
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<tr>
<td>18:2n-6</td>
<td>22.4 (1.26)</td>
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<tr>
<td>18:3n-3</td>
<td>0.13 (0.007)</td>
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<tr>
<td>18:3n-6</td>
<td>0.23 (0.01)</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>0.07 (0.02)*</td>
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<tr>
<td>20:3n-6</td>
<td>0.12 (0.01)</td>
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<tr>
<td>20:4n-6</td>
<td>18.82 (0.92)</td>
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<td>20:5n-3</td>
<td>0.07 (0.001)</td>
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<td>22:5n-3</td>
<td>0.29 (0.01)</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1.4 (0.06)</td>
</tr>
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</table>

*See Table 1 for full names of fatty acids.


