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Production of *cis*-9,*trans*-11-Conjugated Linoleic Acid in Camelina Meal and Okara by an Oat-Assisted Microbial Process

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A method to obtain *cis*-9,*trans*-11-conjugated linoleic acid (*c*9,*t*11-CLA) into camelina meal and okara, the byproducts of plant oil processing, is described. The triacylglycerols in these materials were hydrolyzed with the aid of lipolytically active oat flour for 3 weeks at a water activity of 0.70. The resulting free linoleic acid was then isomerized predominantly to *c*9,*t*11-CLA by resting cells of *Propionibacterium freudenreichii* ssp. *shermanii* in 5% aqueous camelina meal and okara slurries. In camelina meal slurries, *c*9,*t*11-CLA content after 21 h of fermentation was 0.83 mg/mL and 96 mg/g of total lipids. In okara slurries, the content of *c*9,*t*11-CLA was 1.1 mg/mL and 78 mg/g of total lipids. Doubling the hydrolysis time in okara increased the subsequent content of *c*9,*t*11-CLA to 1.4 mg/mL, corresponding to 110 mg/g of total lipids. After isomerization, CLA was concentrated into a particulate material of the slurries by acidification. The results suggest that the method is applicable to a wide spectrum of lipid-containing plant materials to further increase their nutritional value.

KEYWORDS: Conjugated linoleic acid; CLA; camelina; okara; oat; *Propionibacterium*

INTRODUCTION

Conjugated linoleic acid (CLA) has various significant biological activities, which are isomer-specific. The *cis*-9,*trans*-11-CLA (*c*9,*t*11-CLA) has been associated with anti-carcinogenic and anti-atherogenic properties and improved immuno function (*1*−*3*). The principal dietary sources of this CLA isomer are the fats in milk and meat of ruminants. However, with the current trend favoring low-fat foods, the supply of CLA via a normal diet is diminishing. Therefore, increasing effort is being paid to make CLA available in a wider spectrum of food constituents (*2*, *4*).

Linoleic acid, the natural precursor of CLA, is a universal component in plant lipids. Certain ruminant and dairy starter bacteria are able to carry out this isomerization reaction (*5*−*7*), thereby offering a basis for microbial processes to obtain CLA into lipid-rich plant materials. The CLA formed in plant materials by such natural processing may bring added value, especially to side products arising from food manufacturing processes.

The availability of free linoleic acid is a prerequisite for the isomerization reaction. Therefore, a preliminary hydrolysis of acyl lipids is required (*8*, *9*). Oat (*Avena sativa* L.) is a representative of plant materials that contains lipolytic activity hydrolyzing triacylglycerols (*10*) and is therefore suited for CLA production. A two-stage process has been described, including hydrolysis of oat lipids and isomerization of the free linoleic acid predominantly into *c*9,*t*11-CLA using resting cells of a *Propionibacterium* strain (*11*). Characteristically for oat, the lipolytic stage could be carried out at a microbially safe water activity of 0.7. Oat has also been used as the hydrolytic agent to obtain free linoleic acid from sunflower oil for CLA formation (*12*). Thus, the oat-assisted microbial process may turn out as a versatile means for producing CLA into various linoleic-acid-containing materials.

Camelina meal and okara are both linoleic-acid-rich industrial byproducts. Camelina meal is the residue from oil extraction from camelina (*Camelina sativa* L.) seeds, and okara is the insoluble residue left in the manufacture of soymilk from soybeans (*Glycine max* L.), by water extraction (*13*, *14*). In addition to linoleic acid, both materials also contain other nutritionally important components, including protein, dietary fiber, and antioxidants (*13*−*17*). This nutritionally favorable consistency makes both camelina meal and okara particularly interesting candidates to be tested as starting materials in an oat-assisted natural microbial isomerization process. With *c*9,*t*11-CLA, these materials might gain attractiveness as healthy materials and find wider applications in food and feed formulations.

The present report describes a study to obtain *c*9,*t*11-CLA into camelina meal and okara. The process uses lipolytically active oat under microbially safe conditions for hydrolysis of lipids in these materials and resting cells of a *Propionibacterium* strain for the isomerization of liberated linoleic acid into *c*9,*t*11-CLA.

MATERIALS AND METHODS

Plant Materials and Lipid Hydrolysis. Camelina meal and okara were obtained from Raisio Ltd. (Finland). Okara was freeze-dried and ground. Dehulled, non-inactivated oat groats, cultivar Belinda, used in the study were obtained from Raisio Ltd. The groats were milled just prior to use. Camelina meal and okara powder were supplemented with 10% (w/w)

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oat flour, and the mixtures were milled (Fritsch Pulverisette 14, sieve ring of 0.5 mm, Idar-Oberstein, Germany). Water activities (a_w) of the mixtures were adjusted to 0.70 by gradually spraying with deionized water and measuring a_w with a water activity meter (Testo AG, Lenzkirch, Germany) during a period of 2 h. The mixtures were stored in airtight polyethene bags at 23 °C. The progress of lipid hydrolysis was monitored by taking samples at 7 day intervals. From these samples, also, the a_w of the mixtures was checked and readjusted if needed. After the lipid hydrolysis period, the mixtures were stored at −80 °C.

Microbial Strain and Culture Conditions. The bacterium used was *Propionibacterium freudenreichii* ssp. *shermanii* DSM 20270. The cells were cultivated at 30 °C for 48 h in lactate growth medium containing per liter 10 g of tryptone (LabM, Bury, U.K.), 5 g of yeast extract (LabM), and 20 g of 50% sodium lactate solution (Merck, Darmstadt, Germany). The pH of the medium was adjusted to 7.0 before sterilization. The bacterial cells were harvested by centrifugation at 5900*g* for 15 min and resuspended in saline containing per liter 8.5 g of NaCl and 1 g of bacteriological peptone (LabM).

Isomerization Reactions. The hydrolyzed camelina meal−oat and okara−oat mixtures were suspended in water to yield slurries containing 5% (w/v) dry material. The slurries were homogenized with an Ultra Turrax for 2 min at 24 000 rpm before autoclave sterilization for 15 min at 121 °C.

The isomerization reaction was performed in a Biostat MD 2 fermentor (B. Braun, Melsungen, Germany) at 30 °C. To 1.0 L of sterilized slurry, a suspension of propionibacterial cells in 35 mL of saline was added to yield a viable cell count of 1×10^{10} CFU/mL of slurry. The stirring rate applied was 150−200 rpm depending upon the viscosity of the slurry. After 20 min, the pH of the slurry was elevated to pH 8.2−8.4 with 3 M NaOH and was automatically maintained at that level with 1 M NaOH during the isomerization. Samples for fatty acid analysis and viable counts were taken at appropriate intervals. Slurry samples for fatty acid analysis containing both the camelina meal−oat or okara−oat materials and the bacterial cells were freeze-dried prior to analysis.

Harvesting of CLA from the Isomerization Slurries. To study the distribution of CLA between the aqueous solvent and particulate material, the pH of the isomerized slurries was adjusted to pH 8.0 or 5.5 with 2 M HCl solution. Thereafter, the particulate and aqueous phases were separated by centrifugation at 5900*g* for 20 min and freeze-dried before fatty acid analysis.

Analytical Methods. In fatty acid analysis, the fatty acids in samples of camelina meal−oat and okara−oat mixtures or in freeze-dried slurry samples were subjected to the methylation procedure described by Suutari et al. (*18*). In this procedure, the fatty acids were saponified with 3.7 M NaOH in 49% methanol at 100 °C for 30 min and then methylated with 3.3 M HCl in 48% methanol at 80 °C for 10 min. The methyl esters were extracted in hexane/methyl-*tert*-butyl ether solution (1:1), and the extract was washed with aqueous alkali. Analysis of the fatty acid methyl esters was performed by a Hewlett-Packard model 6890 gas chromatograph using a HP-FFAP column (25 m, 0.2 mm inner diameter, 0.33 μm film thickness) with a flame ionization detector. The column temperature was programmed from 70 to 200 °C at a rate of 25 °C/min, and the total run time was 30 min. Nonadecanoic acid methyl ester (Sigma) was added to the samples as an internal standard.

To analyze the amount and fatty acid composition of major lipid classes, the lipids were extracted from samples in dichloromethane/methanol (2:1) twice for 2 h. The extracts were combined and evaporated to dryness under nitrogen. The lipids were separated into the major classes by thin-layer chromatography (*19*). A mixture containing known amounts of triheptadecanoin (Sigma), dipentadecanoin (Sigma), heptadecanoic acid (Sigma), and dipentadecanoyl phosphatidylcholine (Sigma) was used as an internal standard. The separated lipid classes were scraped off from thin-layer plates and used for fatty acid analysis as described above. The degree of triacylglycerol hydrolysis (DH) was calculated as the proportion of free fatty acids from the sum of fatty acids in triacylglycerols and free fatty acids.

Viable counts of the *Propionibacterium* were determined by the pour plate method on sodium lactate agar containing per liter 10 g of yeast extract (LabM), 5 g of tryptone (LabM), 10 g of β-glycerophosphate (Merck), 17 mL of 50% sodium lactate solution (Merck), and 12 g of agar (LabM). The plates were incubated anaerobically (Anaerocult A, Merck) at 30 °C for 6 days.

Table 1. Fatty Acid Composition and Lipid Class Distribution in Camelina Meal and Okara[a]

	camelina meal	okara
fatty acid composition (%)		
palmitic acid	8.1 ± 0.1	11.3 ± 0.1
stearic acid	2.1 ± 0.1	2.1 ± 0.1
oleic acid	14.6 ± 0.2	30.0 ± 0.3
linoleic acid	23.6 ± 0.3	50.7 ± 0.3
α-linolenic acid	39.9 ± 1.2	5.3 ± 0.1
eicosenoic acid	11.7 ± 1.2	0.6 ± 0.1
lipid class distribution (%)		
triacylglycerols	74.8 ± 1.2	92.1 ± 0.2
diacylglycerols	3.7 ± 0.2	1.5 ± 0.1
free fatty acids	9.0 ± 0.9	0.8 ± 0.1
polar lipids	12.5 ± 0.2	5.6 ± 0.3

[a] Results are means ± SD (n = 4).

Statistical Methods. The results are reported as the means and standard deviations (SDs). Student's t test was used to evaluate statistically significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Formation of Free Linoleic Acid from Lipids of Camelina Meal and Okara. The camelina meal and okara powder used differed considerably in their total fatty acid and lipid class compositions (**Table 1**). In camelina meal, the total lipid content was 143 mg/g of dry matter, from which linoleic acid contributed for 34 mg/g, while in okara, the corresponding values were 240 and 122 mg/g, respectively.

The free linoleic acid used for subsequent isomerizarion was obtained by supplementing both camelina meal and okara with lipolytically active oat flour to 10% (w/w) concentration. The total lipid content of the oat flour used was 93 mg/g, from which 38 mg/g was linoleic acid. Thus, the contribution of oat-based linoleic acid to total linoleic acid content remained 11% in camelina meal and 3.3% in okara.

The oat flour was uniformly mixed with camelina meal and okara, and the water activity was adjusted to a_w of 0.70, corresponding to a moisture content of 12.5 and 11.5%, respectively. At this a_w level, lipid hydrolysis has been found to progress in non-inactivated oat without the growth of indigenous microflora (*12*). Free linoleic acid was formed at rates of 1.1 and 1.5 mg/g of dry matter per day in camelina meal and okara, respectively (**Figure 1**). Because of the lower lipid content in camelina meal, the formation of free linoleic acid ceased after 3 weeks. Then, the content of free linoleic acid in camelina meal and okara was 21 and 25 mg/g of dry matter, respectively, and the degree of triacylglycerol hydrolysis (DH) was 66% in camelina meal and 26% in okara (**Figure 2**). According to a previous study, in plain oat flour at a_w of 0.70, the hydrolysis progressed at a rate of 5.8 mg/g of dry matter per day, reaching DH of 55% during the early 3 day phase, and approached 90% after 14 days (*12*). These figures suggest that, despite the low water activity, oat is efficient in liberating free linoleic acid from lipids of other sources. The presence of oat flour was tested to be a prerequisite for the lipid hydrolysis to proceed within the 3 week period at a_w of 0.70 (data not shown). After the hydrolysis, the compositions of free fatty acids closely resembled the total fatty acid compositions before the hydrolysis.

Production of *c*9,*t*11-CLA in Aqueous Slurries of Hydrolyzed Camelina Meal. The camelina meal hydrolyzed with oat flour for 3 weeks was mixed in water to form a 5% dry weight concentration, sterilized, and subjected to isomerization by resting cells of

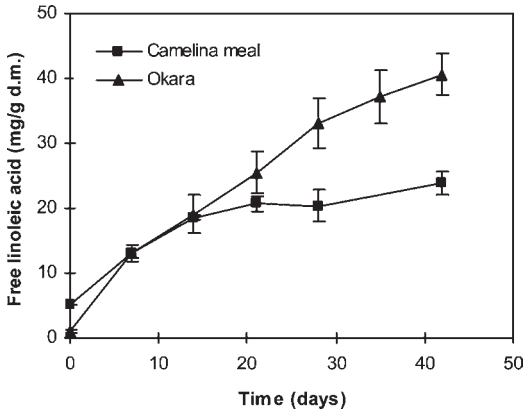

Figure 1. Formation of free linoleic acid in camelina meal and okara supplemented with lipolytically active oat flour (10%, w/w) at a_w of 0.70. The results are means ± SD (n = 4).

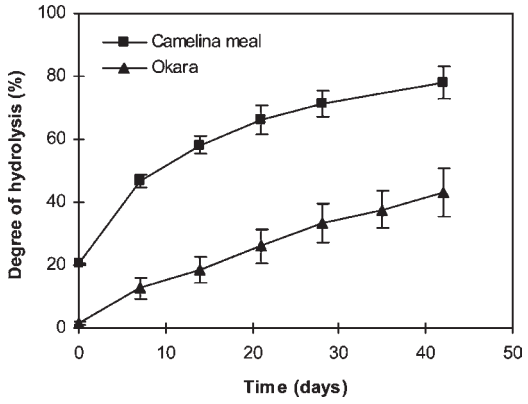

Figure 2. Progress of triacylglycerol hydrolysis in camelina meal and okara supplemented with lipolytically active oat flour (10%, w/w) at a_w of 0.70. The results are means ± SD (n = 4).

P. freudenreichii ssp. *shermanii* DSM 20270. After a short lag phase, the isomerization progressed rapidly. The average *c*9, *t*11-CLA production rate was 0.11 mg/mL per hour during the period of 4−8 h. The final content of *c*9,*t*11-CLA produced at 21 h was 0.83 mg/mL isomerization slurry, corresponding to 16 mg/g of dry matter and 96 mg/g of total lipids (**Figure 3**). Also, the *t*10, *c*12 isomer of CLA was formed, but its content remained 7.5% of the content of *c*9,*t*11-CLA. During the 21 h isomerization period, 94% of the initial free linoleic acid was consumed. These values favor the view that camelina meal is well-suited for microbial production of *c*9,*t*11-CLA. Earlier data indicate that, in plain oat slurry (5% dry weight) containing approximately the same level of initial free linoleic acid and resting *Propionibacterium* cells, 40% of free linoleic acid was converted to CLA and the amount of *c*9, *t*11-CLA formed was 0.45 mg/mL slurry (*12*). This comparison suggests that, in the camelina meal slurry, free linoleic acid is either more quantitatively available for the *Propionibacterium* cells or its antimicrobial activity is attenuated by this material.

Production of *c*9,*t*11-CLA in Aqueous Slurries of Hydrolyzed Okara. Okara−oat mixtures hydrolyzed for 3 weeks with the same mixing ratio as with camelina meal were subjected to isomerizations similar to the camelina meal mixtures. The isomerization initiated more rapidly than in camelina meal slurries, with an average *c*9,*t*11-CLA production rate of 0.14 mg/mL per hour during the first 6 h. Then, the *c*9,*t*11-CLA content was already 0.84 mg/mL isomerization slurry, and after a 21 h isomerization period, the content of *c*9,*t*11-CLA produced was

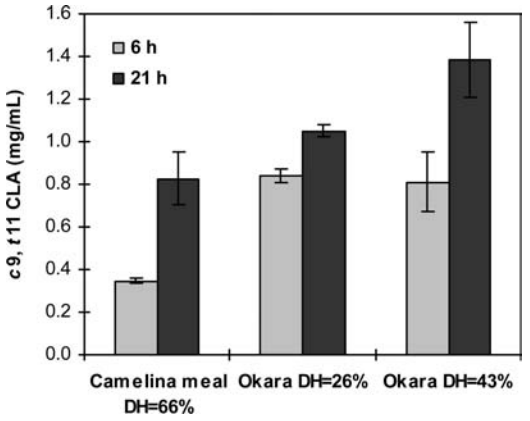

Figure 3. Production of *c*9,*t*11-CLA by *P. freudenreichii* ssp. *shermanii* resting cells in 5% (w/v) aqueous slurries prepared from camelina meal and okara. Prior to fermentation, linoleic acid was released from the materials by mixing them with lipolytically active oat flour (10%, w/w) and adjusting a_w of the mixtures to 0.70. DH = degree of triacylglycerol hydrolysis. The results are means ± SD (n = 4).

1.1 mg/mL, corresponding to 22 mg/g of dry matter and 78 mg/g of total lipids (**Figure 3**). During the isomerization period, 99% of initial free linoleic acid was consumed. Thus, in okara slurries, the conditions for efficient CLA production were even more favorable than in corresponding slurries of camelina meal and more *c*9, *t*11-CLA was formed ($p < 0.05$). For comparison, in a previous study (*8*), the highest CLA content obtained was 2.2 mg/g of lipid corresponding to 0.02 mg/mL when chemically hydrolyzed soy oil in nonfat milk medium was used by growing *P. freudenreichii*.

Effect of the Prolonged Hydrolysis on *c*9,*t*11-CLA Production in Okara Slurries. Because practically all free linoleic acid in okara slurries was consumed during the 21 h isomerization process, it was of interest to test whether the content of *c*9,*t*11-CLA in okara could be further increased by elevating the free linoleic acid concentration. For this purpose, the hydrolysis of okara was continued for an additional 3 weeks at a_w of 0.70. Thereafter, DH was 43%, and the content of free linoleic acid was 41 mg/g of dry matter (**Figures 1** and **2**). When this mixture was subjected to isomerization, *c*9,*t*11-CLA was formed at a rate of 0.13 mg/mL per hour during the first 6 h. After the reaction ceased at 21 h, the concentration of *c*9,*t*11-CLA produced was 1.4 mg/mL isomerization slurry, corresponding to 29 mg/g of dry matter and 110 mg/g of total lipids (**Figure 3**). From the initial free linoleic acid, 78% was consumed.

These results in okara slurries indicate that the DH value correlated with *c*9,*t*11-CLA content (**Figure 3**). Linoleic acid in triacylglycerols was not consumed during the isomerization process even when free linoleic acid in okara was exhausted. In a previous study (*8*), unhydrolyzed soy oil in milk model systems did not enable CLA formation by *P. freudenreichii*. Also, with sunflower oil without a preceding hydrolysis as the starting material, the amount of CLA formed in MRS broth by *P. freudenreichii* was only 0.08 mg/mL, corresponding to 7 mg/g of lipid (*20*). This leaves open the possibility that the strain used possessed weak lipolytic activity or the CLA was formed from free linoleic acid, typically present in low amounts in plant oils.

Recovery of CLA from the Aqueous Slurries. The alkaline isomerization slurries were dispersions where the CLA formed together with other long-chain fatty acids was distributed between aqueous and particulate phases. This distribution is probably dependent upon the composition of the substrate material and, therefore, might form a material-specific challenge for the concentration of CLA from the low dry weight slurries. Because,

Table 2. Effect of pH on the Binding of *c*9,*t*11-CLA into Particulate Material in Aqueous Isomerized Slurries[a]

isomerized slurry	pH 8.0	pH 5.5
camelina meal, DH[b] = 66%	86.2 ± 6.3	100.0 ± 0.0
okara, DH = 26%	50.3 ± 1.1	100.0 ± 0.0

[a] Results are expressed as a proportion of *c*9,*t*11-CLA in particulate material (%) from total *c*9,*t*11-CLA. Results are means ± SD (n = 3). [b] DH = degree of triacylglycerol hydrolysis.

Table 3. Effect of the Slurry Material and Free Linoleic Acid Concentration on the Decrease in Viable Counts of *Propionibacterium* Cells during 21 h of Isomerization[a]

isomerization slurry	initial FLA[b] (mg/mL)	decrease in viable counts (log CFU/mL)
camelina meal, DH[c] = 66%	0.87 ± 0.05	0.20 ± 0.01
okara, DH = 26%	1.31 ± 0.08	0.12 ± 0.03
okara, DH = 43%	1.91 ± 0.06	0.29 ± 0.05

[a] Initial viable counts were 10.0 log CFU/mL. Results are means ± SD (n = 4). [b] FLA = free linoleic acid. [c] DH = degree of triacylglycerol hydrolysis.

however, the particulate material could be easily separated from the aqueous phase, it was of interest to test the possibility to attach CLA into the particulate phase by pH adjustments. In okara slurries, CLA was almost equally distributed between aqueous and particulate phases at the final isomerization pH of 8.0. However, when pH of the slurries was lowered to the acidic side before phase separation, CLA was bound completely to the particulate phase (**Table 2**). In camelina meal slurries, a majority of CLA appeared to be attached to the particulate phase already at pH 8.0. Thus, acidification offers a convenient means to concentrate CLA into the particulate phase from multiphase isomerization slurries.

The particulate material obtained from the phase separation contains also most of the *Propionibacterium* cells. This might add to the value of the particulate CLA preparation because several probiotic characteristics have been attributed to various strains of *P. freudenreichii* (*21*−*24*). Therefore, it was of interest to elucidate the survival of the nongrowing *Propionibacterium* cells during the isomerization process. The viable counts at the beginning of the isomerization and after 21 h indicated that during this period the losses in viability were very small in camelina meal and okara slurries (**Table 3**). However, Rainio et al. (*25*) have reported that, by using micellar linoleic acid instead of plant-based slurries for CLA production, the viable counts of resting *P. freudenreichii* cells decreased 1.6 log units within 21 h when the initial free linoleic acid content was 0.5 mg/mL. Against this background, the multiphase slurries of lipid-containing plant materials can be considered favorable also with respect to the viability of the resting cells.

The present study based on the use of camelina meal and okara suggests that the method combining lipid hydrolysis, microbial isomerization, and CLA harvesting stages has potential as a generally applicable means to enhance the nutritional value of plant materials.

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