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Enrichment of Conjugated Linoleic Acid in Oats (*Avena sativa* L.) by Microbial Isomerization

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A method for microbial isomerization of oat linoleic acid to conjugated linoleic acid (CLA) was developed. The method includes hydrolysis of oat lipids in aqueous flour slurries by the endogenous oat lipase. Then, the flour slurry containing free linoleic acid is utilized as a substrate for the isomerization reaction carried out by resting cells of *Propionibacterium freudenreichii* ssp. *shermanii*. The isomerization reaction progressed most effectively when, after the lipid hydrolysis period, the pH of the slightly acidic oat slurry was elevated to 8.0−8.5 and maintained at this range. With slurries containing 5% (w/v) oat flour, the amounts of CLA formed per dry matter were up to 10.1 mg/g corresponding to 102 mg/g lipids or 0.44 mg/mL slurry. Increments in the flour content up to 15% increased the volumetric production of CLA to 0.85 mg/mL. The proportion of the *cis*-9,*trans*-11 isomer was 80% of the total CLA formed. CLA could be concentrated into the solid material of the oat slurry by acidification.

KEYWORDS: Conjugated linoleic acid; *Avena sativa*; *Propionibacterium freudenreichii*; linoleic acid

INTRODUCTION

Conjugated linoleic acid (CLA) has raised considerable interest due to its several potentially positive effects on human health (*1*−*3*). As an alternative to the synthetic production of CLA, the microbial isomerization of free linoleic acid to CLA is under active research. Several bacteria, including dairy lactic acid and propionic acid bacteria, are capable of carrying out this transformation reaction. In addition, the isomeric composition of the CLA formed by a microbial process is desirable, consisting predominantly of the *cis*-9,*trans*-11 isomer shown to have anticarsinogenic properties in experimental animals (*4*−*6*).

However, the facts that linoleic acid is poorly water soluble and antimicrobial already at very low concentrations (*7*, *8*) enable only very low substrate concentrations to be used in the microbial isomerization processes. This has made the microbiological production alternatives less feasible in industrial scale. To overcome these problems, the dosage of linoleic acid has been increased by adding it as mixed detergent micelles or as complexed to proteins (*9*, *10*). Thus, the effective pool of linoleic acid contacting the cells could be maintained at a nontoxic level allowing the formation of CLA to continue more efficiently (*11*, *12*).

In the microbial isomerization studies reported so far, purified linoleic acid has been added into reaction mixtures tailored suitable for the isomerizing organism. An attractive alternative would be the enrichment of CLA directly in linoleic acid-rich natural food materials by addition of the isomerizing organism to the food matrix. Because only free acid is the substrate for the isomerization, ideal foods would be those containing an endogenous lipolytic activity.

Oats contain higher levels of lipids than most other common cereal grains. Its lipid content varies in the range of 30−120 mg/g dry matter, depending on genetic and environmental factors. Oat lipids are highly unsaturated and contain a considerable proportion of linoleic acid, 25−52% of total fatty acids (*13*). Oats are also known for high endogenous lipase activity, which causes rapid hydrolysis of storage lipids into free fatty acids in milled or damaged oats, even at low water activity levels (*14*). Furthermore, the nutritionally beneficial properties of oats, derived from its soluble fiber, antioxidant and unsaturated fatty acid content, and amino acid composition, are well-documented (*15*−*17*). These features make oats as a highly interesting material to be utilized also in novel microbial processes.

This study describes a method by which the ability of a propionic acid bacterium strain to carry out, with high efficiency, the isomerization reaction can be used to convert oat linoleic acid into its specific health-promoting conjugated isomer.

MATERIALS AND METHODS

Oat Material. Nonheat-treated naked oat groats (*Avena sativa* L. nuda), cultivar Lisbeth, were used in the study. The total fatty acid content of the oat material was 97 mg/g dry matter, of which 40% was linoleic acid (39 mg/g dry matter). The milled flour was suspended in water to yield slurries containing 5−20% (w/v) flour. The slurries were homogenized with an Ultra Turrax for 2 min before use.

Microbial Strain and Culture Conditions. The bacterium used was *Propionibacterium freudenreichii* ssp. *shermanii* strain JS (DSM 7067).

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The cells were cultivated at 30 °C for 54−72 h in a growth medium containing per liter 20 g of whey permeate (Valio Ltd., Helsinki, Finland), 10 g of yeast extract (LabM, Bury, England), and 5 g of tryptone (LabM). The pH of the medium was adjusted to 6.8 before sterilization. The bacterial cells were harvested by centrifugation at 5900*g* for 20 min and resuspended in saline containing per liter 8.5 g of NaCl (LabM) and 1 g of bacteriological peptone (LabM).

Experimental Procedure. Both the lipid hydrolysis and the isomerization reactions were carried out in 250 mL Erlenmeyer flasks containing 100 mL of homogenized oat flour slurry. The cells of *P. freudenreichii* ssp. *shermanii* in a small volume of saline were added to the slurry to yield a viable cell count of 1×10^{10} CFU/mL of slurry. The pH of the slurry was adjusted to pH 7.0 with 1 M NaOH. The incubation temperature was 25 °C. During the lipid hydrolysis period (lasting 2−17 h), the pH of the slurry was not controlled. Following the hydrolysis period, the isomerization period was started by elevation of the pH to alkaline range with 3 M NaOH according to the study plan. During the subsequent 8 h, the pH was adjusted manually at 1−1.5 h intervals. After that, no pH control was applied.

Experiments were carried out also in a Biostat MD 2 fermentor (B. Braun, Melsungen, Germany) with a working volume of 1500 mL at 25 °C with a stirring at 100 rpm. During the isomerization period (30 h), the pH of the oat slurry was automatically maintained at pH 8.5 with 1 M NaOH.

To study the effect of pH on binding of CLA into solid oat material, the pH of the fermented oat slurry was adjusted to pH values of 4.5−9.5 with HCl or NaOH solutions, and the solid 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 freeze-dried oat slurry samples were subjected to the methylation procedure described by ref *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 an HP-FFAP column (25 m, 0.2 mm i.d., 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. Heptadecanoic acid methyl ester (Sigma, St. Louis, MO) was added to freeze-dried samples as an internal standard. The reported values are the means of four measurements (standard deviation < 5%).

For analyzing the amount and fatty acid composition of major lipid classes, the lipids were extracted from freeze-dried oat slurry 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 as described by ref *19*. A mixture containing known amounts of dipentadecanoyl phosphatidylcholine (Sigma), heptadecanoic acid (Sigma), dipentadecanoin (Sigma), and triheptadecanoin (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 lipid hydrolysis was calculated as the proportion of free fatty acids from the sum of fatty acids in triacylglyserols and as free acids. The reported values are the means of 2−4 measurements (standard deviation < 5%).

Viable counts of the propionic acid bacterium were determined by plating on sodium lactate agar containing per liter 10 g of yeast extract (LabM), 5 g of tryptone (LabM), 10 g of β-glycerophosphate (Merck, Darmstadt, Germany), 17 mL of 50% (v/v) sodium lactate (Merck), and 12 g of agar (LabM). The plates were incubated anaerobically at 30 °C for 6 days.

RESULTS

Lipid Hydrolysis and Formation of CLA in Aqueous Oat Slurry. For the production of CLA in aqueous oat slurries, the substrate, free linoleic acid, was first allowed to be liberated from oat acyl lipids by the lipase naturally present in oats. Then, the isomerization of the free linoleic acid was carried out by the cells of *P. freudenreichii* ssp. *shermanii*.

Table 1. Fatty Acid Content (mg/g Dry Matter) of 5% (w/v) Oat Flour Slurry during the Overall CLA Production Process Consisting of Periods of Lipid Hydrolysis and Microbial Isomerization by *P. freudenreichii* ssp. *shermanii*[a]

	hydrolysis period		isomerization period			
fatty acid	0 h	17 h	2 h	4 h	6 h	30 h
palmitic acid	19.2	17.6	18.9	18.9	18.5	17.7
stearic acid	1.0	1.0	1.0	1.0	1.0	1.0
oleic acid	45.4	42.2	44.5	44.5	43.5	39.3
linoleic acid	45.6	40.4	37.4	35.6	33.8	29.7
free linoleic acid	4.6	12.6	*b*	*b*	*b*	2.9
linolenic acid	1.2	1.0	0.8	0.8	0.8	0.7
CLA	0.7	1.2	6.2	8.1	8.8	10.1

[a] The isomerization period was performed at a constant pH of 8.5. [b] Blank entries were not analyzed.

As the oat lipid hydrolysis and the microbial isomerization favor different pH environments, these two reactions were performed consecutively, by adjusting the pH of the oat slurry. The hydrolysis was done in the neutral or slightly acidic pH range, whereas the isomerization reaction was conducted under alkaline conditions.

The hydrolysis period was initiated by adjusting the pH of the oat slurry to 7.0. During progress of the hydrolysis, the pH decreased typically to 4.7−4.8. After a hydrolysis period of 17 h, 28−56% of the acyl linoleic acid in oat had been hydrolyzed to free acid corresponding to 12−22 mg free linoleic acid per g dry matter.

Also, shorter lipid hydrolysis periods were tested, but for example, after 4 h, the amount of free linoleic acid was only 55% of that formed during the 17 h hydrolysis. This led to the adoption of the overnight (17 h) hydrolysis in the routine processes.

After the hydrolysis period, the pH of the acidic oat suspension was elevated to a slightly alkaline range (8.0−8.5) and maintained there by manual pH control. Then, the cells of the propionic acid bacterium added already prior to the initiation of the hydrolysis period rapidly began to form CLA concomitantly with a decrease in linoleic acid concentration (**Table 1**).

Lipid class analysis of the CLA-enriched oat slurries revealed that 88−92% of the CLA formed was present as free acid while the rest was bound to triacylglycerols. The proportion of the *cis*-9,*trans*-11 isomer was 80% of the total CLA formed.

No CLA was formed if the propionic acid bacterium cells were omitted from the oat slurries even though the pH was increased to 8.0 after the 17 h hydrolysis period. This indicates the essential role of the propionic acid bacterium for the isomerization reaction to occur. However, the propionic acid bacterium cells did not grow during either the hydrolysis or the isomerization period. The viable propionic acid bacterium counts log CFU/mL were 10.08 ± 0.04 ($n = 4$) throughout the hydrolysis period and 9.92 ± 0.03 at the end of the isomerization period at pH 8.5. Thus, the isomerization of linoleic acid to CLA in oat slurries occurred by nongrowing cells of *P. freudenreichii* ssp. *shermanii*.

Effect of pH on Isomerization of Linoleic Acid. Because pH appeared to be the critical factor for the efficiency of isomerization, its effect was studied at various pH values between 7.0 and 9.5. The pH of the oat slurry was maintained at the desired levels by manual control and appropriate adjustments at intervals of 1−1.5 h. Between the control points, the pH decreased by about 0.5 pH units. In oat slurries where

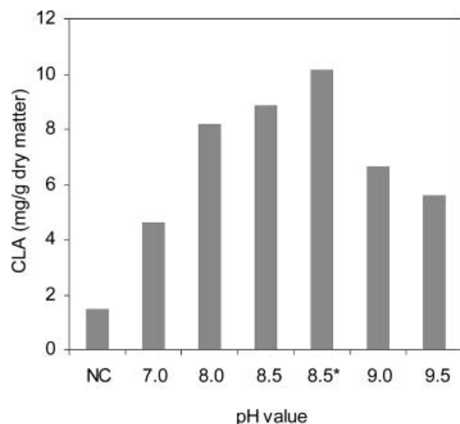

Figure 1. Effect of pH during the isomerization period on the amount of CLA formed by *P. freudenreichii* ssp. *shermanii* in aqueous oat slurry. The pH was maintained at the desired value by manual adjustments at intervals of 1–1.5 h. For comparison, automatic control of pH at 8.5 was performed in a fermentor (8.5*). NC, no pH control.

pH was not adjusted during the isomerization period, the pH decreased to about 4.4.

The optimum pH range for the isomerizarion reaction was 8.0−8.5 (**Figure 1**). At that pH range, the amount of CLA formed in 24 h was 8.2−8.8 mg/g dry matter, corresponding to 0.35−0.39 mg/mL of 5% oat slurry or 77−85 mg/g lipids. At pH 9.0−9.5, the formation of CLA was as rapid as at pH 8.5 during the first 4 h but ceased thereafter (data not shown).

To make the isomerization reaction even more efficient, it was carried out in a fermentor with automatic and continuous pH control. Because of the more stable pH level, 15% more CLA (10.1 mg/g dry matter corresponding to 102 mg/g lipids) was produced than with the manual pH control described above (**Figure 1**). Regardless of how the pH control was done, the rate of CLA formation was highest during the initial stages after the elevation of pH. For example, during the first 2 h of isomerization in the fermentor, CLA was formed 2.5 mg/g dry matter per hour (**Table 1**). Then, the total yield of CLA from the free linoleic acid present at the end of the hydrolysis period in 5% flour slurry was 71%.

Effect of Free Linoleic Acid Content. Because oat flour was the only source of free linoleic acid, the efficiency of volumetric CLA production expectedly depended on the flour concentration in the slurries. On the other hand, flour concentration affects also the degree of lipid hydrolysis and, thereby, the subsequent isomerization reaction. Therefore, the efficiency of the CLA production process was tested when the degree of lipid hydrolysis was varied by varying the flour content between 5 and 20% (w/v).

The degree of hydrolysis with flour contents of 5, 10, 15, and 20% was 50, 61, 72, and 82%, respectively, after a 17 h hydrolysis period. Thus, the amount of free linoleic acid could be increased by increasing the flour content. Also, the total amount of CLA formed during the isomerization period increased concomitantly until the content of free linoleic acid reached a value of 3.3 mg/mL (degree of hydrolysis 72%) (**Figure 2**). The amount of propionic acid bacterium cells applied was the same independent of the varying flour content. This demonstrates the high potency of the bacterial cells used to carry out the isomerization reaction in aqueous oat environments. At the two highest flour contents tested, 15 and 20%, where the amount of free linoleic acid was 3.3 and 4.1 mg/mL, respectively, the amount of CLA formed was 0.85 mg/mL.

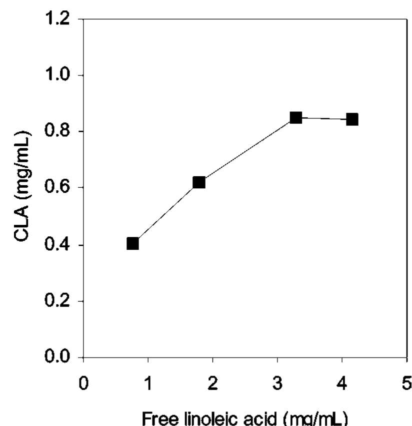

Figure 2. Effect of free linoleic acid content on CLA formed by *P. freudenreichii* ssp. *shermanii* in aqueous oat slurry containing different amounts of oat flour at pH 8.5. Linoleic acid was liberated from oat lipids by the action of endogenous oat lipase.

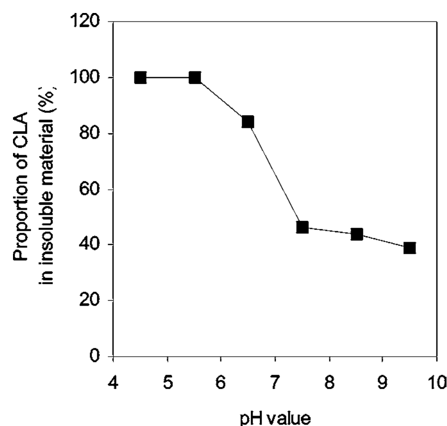

Figure 3. Distribution of CLA between aqueous and solid phases in hydrolyzed and isomerized oat slurry adjusted to various pH values.

Recovery of CLA from the Aqueous Oat Slurry. The CLA formed in the alkaline oat slurries was distributed between the aqueous and the solid phases. By acidification of the hydrolyzed and isomerized oat slurry, the solubility of CLA in the aqueous phase could be decreased and thereby its adherence to the solid phase increased (**Figure 3**). At pH 5.5 or below, virtually all of the CLA was adhered to the solid material consisting of insoluble flour particles and the bacterial cells. This material could be harvested by mere sedimentation or centrifugation at low *g* values.

DISCUSSION

The present process aimed at the enrichment of CLA in food materials includes two essential stages, hydrolysis of triacylglycerols and microbial isomerization of the liberated linoleic acid. Therefore, linoleic acid-rich food materials containing lipolytic activity are applicable as such for the enrichment process. In this respect, oats are especially suitable among the common cereals since the endogenous lipase activity initiates lipid hydrolysis instantaneously when groats are milled and contacted with water (*14*). Lipase activity can be raised also in other cereals, e.g., by germination. Different malted cereals would therefore represent interesting alternatives for the elevation of their CLA content via the present fermentation process.

Even though the amounts of CLA formed were high in comparison to existing microbiological methods, still 28−85% of the free linoleic acid present at the end of the hydrolysis

period was left after the isomerization period. The degree of linoleic acid isomerization was dependent on the flour content and pH of the slurry. This may be explained by distribution of free linoleic acid between different phases with different availability toward the bacterial cells. In the complex slurry, free linoleic acid binds to proteins and other flour components, e.g., amylose (*20*, *21*). Some of these interactions may, under the present process conditions, retain free linoleic acid unavailable to the bacterial cells. Apparently, these interactions can be reduced by alkaline pH, which increases the water solubility of linoleic acid. Microbial isomerizations of linoleic acid in other, noncereal milieu have been reported to occur also in the slightly acidic pH region (*11*, *12*).

In the present process for CLA production, the previously reported antimicrobial effect of linoleic acid (*8*, *9*) was attenuated, probably due to binding of part of the free linoleic acid to soluble, partly soluble, and insoluble components of oats (*22*) so that the linoleic acid pool contacting the cells did not rise to an inhibitory level. Furthermore, dispersing or solubilizing agents, which might limit the use of the product as a food or complicate the recovery of CLA after the isomerization, were not required. Moreover, oat slurries probably reduce the adherence of CLA to the cells, a phenomenon found to limit the duration of the isomerization reaction with micellar linoleic acid substrates (*23*).

The propionic acid bacterium strain used was of dairy origin (*24*). Against this background, its applicability in aqueous oat milieu is noticeable. The efficiency of the resting cells in oat slurries was comparable to that found with the same amount of cells in a whey-based medium supplemented with micellar linoleic acid (*11*), as judged from the volumetric production efficiencies from 0.6 mg free linoleic acid/mL, 0.44 and 0.52 mg CLA/mL, respectively. Although the oat slurry did not support growth of the organism, its slightly slimy consistency probably offered a protective environment for the cells.

The present microbial method, being based on the use of oat slurries as the source of linoleic acid, offers several advantages over currently reported microbial methods for CLA production. It is also an example that a natural and healthy food material can be further enriched with a health beneficial fatty acid by a natural microbial process. The method described forms a basis for the production of, e.g., novel oat-based functional foods.

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LITERATURE CITED

(1) Belury, M. A. Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action. *Annu. Rev. Nutr.* **2002**, *22*, 505−531.
(2) Kritchevsky, D. Antimutagenic and some other effects of conjugated linoleic acid. *Br. J. Nutr*. **2000**, *83*, 459−465.
(3) Pariza, M. W.; Park, Y.; Cook, M. E. The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* **2001**, *40*, 283−298.
(4) Kishino, S.; Ogawa, J.; Omura, Y.; Matsumura, K.; Shimizu, S. Conjugated linoleic acid production from linoleic acid by lactic acid bacteria. *J. Am. Oil Chem. Soc.* **2002**, *79*, 159−163.
(5) Verhulst, A.; Janssen, G.; Parmentier, G.; Eyssen, H. Isomerization of polyunsaturated long chain fatty acids by propionibacteria. *Syst. Appl. Microbiol.* **1987**, *9*, 12−15.
(6) Lavillonnière, F.; Chajès, V.; Martin, J.-C.; Sébédio, J.-L.; Lhuillery, C.; Bougnoux, P. Dietary purified *cis*-9,*trans*-11 conjugated linoleic acid isomer has anticarsinogenic properties in chemically induced mammary tumors in rats. *Nutr. Cancer* **2003**, *45*, 190−194.
(7) Nieman, C. Influence of trace amounts of fatty acids on the growth of microorganisms. *Bacteriol. Rev*. **1954**, *18*, 147−163.
(8) Boyaval, P.; Corre, C.; Dupuis, C.; Roussel, E. Effects of free fatty acids on propionic acid bacteria. *Lait* **1995**, *75*, 17−29.
(9) Rainio, A.; Vahvaselkä, M.; Suomalainen, T.; Laakso, S. Reduction of linoleic acid inhibition in production of conjugated linoleic acid by *Propionibacterium freudenreichii* ssp. *shermanii*. *Can. J. Microbiol.* **2001**, *47*, 735−740.
(10) Ogawa, J.; Matsumura, K.; Kishino, S.; Omura, Y.; Shimizu, S. Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* **2001**, *67*, 1246−1252.
(11) Rainio, A.; Vahvaselkä, M.; Suomalainen, T.; Laakso, S. Production of conjugated linoleic acid by *Propionibacterium freudenreichii* ssp. *shermanii*. *Lait* **2002**, *82*, 91−101.
(12) Jiang, J.; Björck, L.; Fondén, R. Production of conjugated linoleic acid by dairy starter cultures. *J. Appl. Microbiol.* **1998**, *85*, 95−102.
(13) Zhou, M.; Robards, K.; Glennie-Holmes, M.; Helliwell, S. Oat lipids. *J. Am. Oil Chem. Soc.* **1999**, *76*, 159−169.
(14) Youngs, V. L. Oat lipids and lipid-related enzymes. In *Oats: Chemistry and Technology*; Webster, F. H., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1986; pp 205−226.
(15) Lásztity, R. Oat grain—a wonderful reservoir of natural nutrients and biologically active substances. *Food Rev. Int*. **1998**, *14*, 99−119.
(16) Anderson, J. W.; Spencer, D. B.; Hamilton, C. C.; Smith, S. F.; Tietyen, J.; Bryant, C. A.; Oeltgen, P. Oat-bran cereal lowers serum total and LDL cholesterol in hypercholesterolemic men. *Am. J. Clin. Nutr.* **1990**, *52*, 495−499.
(17) Zarkadas, C. G.; Hulan, H. W.; Proudfoot, F. G. A comparison of the amino acid composition of two commercial oat groats. *Cereal Chem*. **1982**, *59*, 323−327.
(18) Suutari, M.; Liukkonen, K.; Laakso, S. Temperature adaptation in yeasts: the role of fatty acids. *J. Gen. Microbiol.* **1990**, *136*, 1469−1474.
(19) Liukkonen, K. H.; Montfoort, A.; Laakso, S. V. Water-induced lipid changes in oat processing. *J. Agric. Food Chem.* **1992**, *40*, 126−130.
(20) Lehtinen, P.; Laakso, S. Antioxidative-like effect of different cereals and cereal fractions in aqueous suspension. *J. Agric. Food Chem*. **1997**, *45*, 4606−4611.
(21) Chung, O. K.; Tsen, C. C. Changes in lipid binding and distribution during dough mixing. *Cereal Chem*. **1975**, *52*, 533−548.
(22) Lehtinen, P.; Laakso, S. Inhibition of linoleic acid oxidation by interaction with a protein-rich oat fraction. *J. Agric. Food Chem.* **2000**, *48*, 5654−5657.
(23) Rainio, A.; Vahvaselkä, M.; Laakso, S. Cell-adhered conjugated linoleic acid regulates isomerization of linoleic acid by resting cells of *Propionibacterium freudenreichii*. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 481−484.
(24) Suomalainen, T. H.; Mäyrä-Mäkinen, A. M. Propionic acid bacteria as protective cultures in fermented milks and breads. *Lait* **1999**, *79*, 165−174.

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