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Forthill, Kinsale, County Cork (IE). **QUIGLEY, Eamonn** [IE/IE]; 3, Woodlands, Cappagh, Kinsale, County Cork (IE). **ROSS, Paul** [IE/IE]; Grange, Kilworth, County Cork (IE). **STANTON, Catherine** [IE/IE]; Grange, Kilworth, County Cork (IE).

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(74) Agent: **GATES, Marie Christina Esther**; Tomkins & Co., 5 Dartmouth Road, Dublin, 6, (IE).

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(71) Applicants (*for all designated States except US*): **UNIVERSITY COLLEGE CORK, NATIONAL UNIVERSITY OF IRELAND, CORK** [IE/IE]; Western Road, Cork (IE). **TEAGASC, - The Agriculture and Food Development Authority** [IE/IE]; 19 Sandymount Avenue, Dublin, 4 (IE). **ALIMENTARY HEALTH LTD.** [IE/IE]; Cork Airport, Business Park, Building 2800, Kinsale Road, Cork (IE).

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(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **O'MAHONY, Liam** [IE/IE]; 5 The Lagan, Riversdale,, Rathcormac, County Cork (IE). **SHANAHAN, Fergus** [IE/IE]; Seafort,

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(54) Title: MODULATION OF TISSUE FATTY ACID COMPOSITION OF A HOST BY HUMAN GUT BACTERIA

(57) Abstract: The current invention provides use of a CLA -producing bacterium for the *in vivo* conversion in the gut of polyunsaturated fatty acids to CLA. The CLA- producing bacterium is selected from one or more of the group consisting of propionibacteria, lactobacilli, lactococci and streptococci, and bifidobacteria.



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Title**Modulation of tissue fatty acid composition of a host by human gut bacteria****Field of the Invention**

The present invention relates to the use of microbial species to modulate the
5 tissue fatty acid composition of a host using human gut bacteria and to convert
polyunsaturated fatty acids to CLA *in vivo*. The invention provides methods and
compositions for use in such methods.

Background to the Invention

The human body can produce all but two of the fatty acids it requires; thus
10 linoleic acid (C18:2 n -6, precursor of n -6 series of fatty acids) and α -linolenic
acid (C18:3 n -3, precursor of n -3 series of fatty acids) are essential dietary fatty
acids. Although mammalian cells cannot synthesize these fatty acids, they can
metabolize them into more physiologically active compounds through a series of
elongation and desaturation reactions, in which linoleic acid is converted to
15 arachidonic acid (C20:4 n -6) and α -linolenic acid is metabolized to EPA
(C20:5 n -3) via the action of Δ^6 desaturase, Δ^5 desaturase and elongase enzymes
(1). The resulting highly unsaturated fatty acid metabolites are necessary for the
functioning of the cell membrane, the proper development and functioning of the
brain and nervous systems, and the production of inflammatory mediators, i.e.
20 eicosanoids (thromboxanes, leukotrienes and prostaglandins) (1, 2). The
eicosanoids derived from arachidonic acid, such as the 2-series prostaglandins
and the 4-series leukotrienes, are in general being ascribed to be proinflammatory
and to exhibit disease-propagating effects if present in abundance (3), whereas
the eicosanoids derived from EPA, such as the 3-series prostaglandins and the 5-
25 series leukotrienes are considered to be less inflammatory or even anti-
inflammatory (3, 4). Thus, increasing the ratio of n -3 to n -6 fatty acids in the
diet, and consequently favouring the production of EPA, the balance of
eicosanoids can be shifted towards a less inflammatory mixture. Conjugated
linoleic acid (CLA) refers to a group of polyunsaturated fatty acids which are
30 positional and geometric isomers of linoleic acid [C18:2 *cis*-9 (*c*9), *cis*-12 (*c*12)
octadecadienoic acid]. CLA is a natural component of milk fat due to the
microbial biohydrogenation of linoleic acid in the rumen. CLA is thus found in
the milk fat and meat of ruminant animals. The predominant CLA isomer found
in nature, and in food, is the *c*9, *t*11 CLA isomer. It has been proposed that CLA

has positive effects on many aspects of human health. Most notably are the effects within the areas of cancer, immune modulation, atherosclerosis and obesity. The mechanisms of action underlying these biological properties are not clearly understood however. CLA was first implicated in down-regulating the generation of inducible eicosanoids (i.e. PGE₂ and LTB₄) involved in early micro-inflammation events, but more recently, CLA has also been shown to modulate the expression of genes regulated by peroxisome proliferator-activated receptors (PPARs). PPARs (α , β/δ , and γ) are ligand-activated transcription factors that increase transcription of target genes by binding to a specific nucleotide sequence in the gene's promoter. Numerous works have shown CLA's anti-inflammatory effect in the gastrointestinal tract, an effect linked to its ability to interfere with proinflammatory intracellular signalling cascades.

The human gut harbours a diverse bacterial community that can comprise more than 1000 different species, out-numbering the human somatic and germ cells 10-fold (5). There is now compelling evidence that the enteric microbiota plays an important role in the health and well-being of the host. For example, evidence obtained from comparative studies of germ-free and conventionally colonised animals have shown that the human enteric microbiota exerts a conditioning effect on intestinal homeostasis, delivering regulatory signals to the epithelium and instructing mucosal immune responses (5, 6). Furthermore, the enteric microbiota was recently established as a regulator of fat storage (7).

Little is known regarding the interplay between members of the human enteric microbiota and fatty acids. However, there are some indications that intestinal bacteria within the gastrointestinal tract (GIT) may interact with different fatty acids. For example, it has been shown that administration of probiotics (*Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* Bb12) to pregnant women affected placental fatty acid composition (8). Moreover, Kankaanpää *et al.* (9) demonstrated that administration of formula supplemented with different probiotics (*B. animalis* subsp. *lactis* Bb12 and *L. rhamnosus* GG) to infants resulted in changes in the fatty acid composition of serum lipids.

Recent studies have also reported that intestinal bacteria of human origin can convert dietary linoleic acid to bioactive isomers of CLA both *in vitro* and *in vivo* (10-13). Some bacteria of marine origin even possess the metabolic capacity to

synthesize EPA and DHA (14-16). In these bacteria, EPA and DHA are synthesized *de novo* by polyunsaturated fatty acid synthase genes rather than by chain elongation and desaturation of existing fatty acids (17, 18). The current inventors have shown that increased concentrations of EPA and DHA were
5 obtained in adipose tissue of mice administered the metabolically active strain *B. breve* NCIMB 702258 compared to unsupplemented mice (unpublished data). It has been demonstrated that bacterial cultures, other than rumen bacteria, possess the ability to generate *c9, t11* CLA from free linoleic acid. These include the intestinal microflora of rats, propionibacteria, lactobacilli, lactococci and
10 streptococci, and bifidobacteria, including a number of strains of human origin. Of particular interest are bifidobacteria, with some clinical studies linking their presence in the gut with specific health effects, including improvement of gastrointestinal disturbances, enhancement of immune function, and cancer suppression. Unlike ruminants, human production of CLA from linoleic acid
15 does not appear to occur at any significant level. The amount of CLA in human adipose tissue is thought to be directly related to dietary intake. The best source of CLA is fat from ruminants, however since consumption of fat from ruminants is usually not recommended by nutritionists (due to its high concentration of saturated fatty acids) the ingestion of a CLA-producing bacteria could be an
20 option for maintaining and supplementing levels of CLA in the gut. CLA has been shown to be generated *in vitro* from linoleic acid, but according to Bassaganya-Riera *et al.* (16) and Kamlage *et al.* (21), this synthesis appears to be inhibited *in vivo*.

Certain metabolic capabilities of microorganisms that easily are observed *in vitro*
25 do not necessarily occur *in vivo*. This appears to be true for enzymatic activities that are not essential for survival of the microorganism such as the biotransformation of non-nutritive dietary compounds such as LA. CLA production has not been described as a mechanism by which probiotics exert anti-inflammatory effects. Several studies have investigated the
30 bioproduction of CLA by various lactobacilli and bifidobacteria but the action of this CLA has not been investigated. Inflammatory bowel disease (IBD) is a widespread and debilitating illness afflicting over 3.5 million people worldwide. It is a chronic disease of the digestive tract, and usually refers to two related conditions of unknown cause, ulcerative colitis and Crohn's disease, which are

characterized by chronic and spontaneously relapsing inflammation leading to destruction of the gut mucosa. These two diseases are important since they are increasing in frequency, disabling for many patients, and generating a significant burden on the health care system. Although the etiology of IBD remains

5 unknown, there is increasing experimental evidence to support a role for luminal bacteria in the initiation and progression of these intestinal conditions; probably related to an imbalance in the intestinal microflora, relative predominance of aggressive bacteria and insufficient amount of protective species. A lot of data supports the theory that these diseases represent the outcome of 3 essential

10 interactive factors: host susceptibility, enteric microflora, and mucosal immunity. Current treatments for IBD include corticosteroids, antibiotics and immunomodulators. Although IBD therapies have improved, they still are only modestly successful for the long-term management of the disease and result in significant side effects. Therefore, exploring novel preventive or therapeutic

15 interventions remains important. A possible therapeutic approach in IBD therapy is the administration of probiotic microorganisms. Probiotics are defined as live microorganisms that confer health benefits to the human host through a number of mechanisms. Probiotic bacteria are attractive alternatives for the treatment of gastrointestinal inflammation due to their effects on the composition of the gut

20 flora and activity on the immune system. Recently, some investigators have reported success with different strains of probiotics in the treatment of chronic intestinal diseases such as ulcerative colitis (5), Crohn's disease (6), and pouchitis (7). *E. coli* (Nissle 1917), the yeast *Saccharomyces boulardii*, *Lactobacillus GG*, and VSL# 3, a cocktail of eight different strains, have been

25 used successfully in human pathology. In addition, indirect evidence demonstrates the potential impact of nutrition in general and lipid nutrition in particular in modulating the course of IBD. For example, in a study by Hontecillas *et al.* (10), conjugated linoleic acid (CLA), a dietary fatty acid, proved to ameliorate IBD in a pig model of bacterial-induced colitis. CLA

30 ameliorated intestinal lesion development, prevented growth suppression and maintained or induced PPAR γ expression while repressing IFN- γ expression. N-3 polyunsaturated fatty acids (PUFA) [i.e., docosahexaenoic (DHA) and eicosapentaenoic (EPA)] are other beneficial fatty acids that elicit potent anti-

inflammatory and immunoregulatory properties (11). In similar action to dietary CLA, n-3 PUFA have been reported to ameliorate intestinal inflammation in animal models of IBD (9).

Object of the Invention

- 5 It is an object of the invention to provide compositions and methods, which can lead to the generation of health-promoting fatty acids within the mammalian body. A further object is to provide compositions and methods, which reduce inflammation in the digestive tract. The methods and compositions may reduce or alleviate the symptoms of inflammatory bowel disease. A still further
- 10 objective is to provide probiotic compositions having the above effects, which can easily be consumed. The probiotic compositions may be foodstuffs or pharmaceutical products. A still further object is to provide methods for the in vivo conversion in the gut of linoleic acid to CLA and methods to alter the fatty acid composition of internal organs of the body. A further object was to provide
- 15 co-administration of commensal bifidobacteria, with ability to produce bioactive isomers of conjugated linoleic acid (CLA) in combination with α -linolenic acid influence the EPA and DHA concentrations of different tissues.

Summary of the Invention

- According to the present invention there is provided use of a CLA-producing
- 20 bacterium in the preparation of a composition for the treatment of Inflammatory Disease. The CLA-producing bacterium may be selected from the group consisting of propionibacteria, lactobacilli, lactococci and streptococci, and bifidobacteria. The bacterium may be *Bif. Breve*, *Bif. Lactis*, *Bif. Dentum*, *Lactobacillus rhamnosus* or *Butyrivibrio fibrisolvens*. In particular, the CLA-
- 25 producing bacterium may be the publicly available strain *Bifidobacterium breve* as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 702258 or *B. breve* DPC 6330 as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41497 on 28th
- September 2007, or *B. longum* DPC 6315 as deposited at the National Culture of
- 30 Industrial and Marine Bacteria under the accession no.41508 on 18th October 2007.

The invention also provides use of a CLA –producing bacterium for the in vivo conversion in the gut of polyunsaturated fatty acids, such as linoleic acid to CLA.

The invention also provides use of a CLA –producing bacterium to alter the fatty acid composition of internal organs of the body. The CLA-producing bacterium may be as defined above.

- A further aspect the invention relates to a probiotic composition comprising a
- 5 CLA producing organism together with pharmaceutically acceptable or nutritionally acceptable additives. The CLA-producing bacterium may be as defined above. The probiotic composition may be a pharmaceutical composition or a foodstuff composition. In the case of a pharmaceutical composition, it may be formulated as a tablet, capsule, suspension, powder of the like, and contain
- 10 pharmaceutically acceptable carriers or excipients, as would be well known in the art. If formulated as a foodstuff, the composition may be a yogurt, a yogurt drink, a cheese, a milk, a spread, a fruit juice, a water which is either flavoured or unflavoured or any other edible composition. This probiotic combination may lead to a more healthy/desirable fatty acid composition of host tissues such as the
- 15 liver where it may protect against non-alcohol –induced fatty liver disease. The probiotic compositions can reduce gut inflammation in diseases such as Inflammatory Bowel Syndrome or Inflammatory Bowel Disease, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease, eczema, asthma or psychiatric diseases such as depression. The composition may also be formulated as an
- 20 animal feedstuff, together with conventional animal feed ingredients. The probiotic composition may further comprise a substrate, which can be converted into a bioactive compound *in vivo* by the CLA producing organism. The substrate may be a polyunsaturated fatty acid, such as, but not limited to linoleic acid, linolenic acid, oleic acid, palmitic acid, or stearic acid.
- 25 In a still further aspect the invention provides a method of converting dietary polyunsaturated fatty acids, such as linoleic acid to CLA *in vivo* comprising administration to a subject of a live CLA-producing bacterial strain. The invention also provides a method of altering the fatty acid composition of internal organs of the body comprising administering to a subject a live CLA-
- 30 producing bacterial strain. Suitable bacterial strains are as defined above. The invention also provides CLA producing strains isolated from the human intestine, *B. breve* DPC 6330, which has highest conversion rate of 76.65 +/- 1.75% conversion of linoleic acid to *c9, t11* CLA, and *B. breve* DPC 6331, which

has 60.12 +/- 5.14% conversion, compared to the publicly available strain *B. breve* NCIMB 702258 which has a conversion rate to *c9, t11* CLA of 60%.

- In a still further aspect the invention provides a method to drive DHA
- 5 (docosahexaenoic acid) /EPA (eicosapentaenoic acid) incorporation into host tissues using dietary CLA or a strain producing CLA, in order to improve memory loss and cognition.

10 **Brief Description of the Drawings**

Fig. 1. Cytokine production by stimulated splenocytes. A-C shows cytokine production following stimulation with antiCD3-antiCD28 monoclonal antibodies, D-E shows cytokine production following stimulation with the proinflammatory bacterium *S. typhimurium* UK1. There was a significant

15 difference in the proinflammatory cytokines IFN- γ , TNF- α and IL-6 in the groups fed *c9, t11* CLA (group 4) and the probiotic *B. breve* NCIMB 702258 (group 3) (*p < 0.05). Results are expressed as mean cytokine levels $\pm$ standard error per group (n=8).

Fig. 2. Body weight development. Values are means $\pm$ SEM (n = 9).

20 **Fig. 3.** Recovery of *B. breve* NCIMB 702258 in stool from probiotic fed mice (group A). Results are expressed as log mean colony forming units (CFU/g) $\pm$ SD. No probiotics were isolated from mice in the placebo group.

Fig. 4. Bioproduction of *c9, t11* CLA by *B. breve* NCIMB 702258 occurs *in vivo*. Group A shows mice fed LA together with *B. breve* NCIMB 702258, group

25 B shows LA-fed mice. Columns with * are statistically significant different from corresponding columns in control group (B).

Fig. 5. *c9, t11* CLA incorporation into faeces harvested after 8 weeks feeding. Group A had a 2.4-fold higher amount of *c9, t11* CLA in the faeces compared to group B (p=0.001). A high level of *c9, t11* CLA in the faeces correlated with a

30 low level of LA (r = -0.863).

Fig. 6. Enumerated *B. breve* NCIMB 702258 in the large intestinal contents. *B. breve* NCIMB 702258 was detected in the large intestine at $\sim 4.6 \times 10^5$ CFU/g in mice that received *B. breve* in combination with α -linolenic acid (group A) and $\sim$

1.4×10⁶ CFU/g in the mice that received *B. breve* without α-linolenic acid (group C) (p>0.05). *B. breve* NCIMB 702258 was not isolated from any of the mice that did not receive *B. breve* (group B and group D). Enumeration of lactobacilli was performed using lactobacilli selective agar (LBS). The numbers of CFU obtained on LBS did not differ between the groups (p>0.05). Results are expressed as log means CFU ± SEM (CFU/g).

Fig. 7. Incorporation of EPA in liver, adipose tissue and brain demonstrating that dietary supplementation with *B. breve* in combination with α-linolenic acid (group A) increases the content of EPA in the liver significantly compared to mice receiving α-linolenic acid without the *B. breve* strain (group B) (p<0.05). A, B, C, D Different superscript numbers within a column indicate significant differences (n=8, p<0.05). EPA is expressed as Mean ± SEM g/100g FAME.

Fig. 8. Incorporation of DHA in liver, adipose tissue and brain demonstrating that dietary supplementation with *B. breve* in combination with α-linolenic acid (group A) increases the content of DHA in the brain significantly compared to mice receiving α-linolenic acid without the *B. breve* strain (group B) (p< 0.05). A, B, C, D Different superscript numbers within a column indicate significant differences (n=8, p<0.05). DHA is expressed as Mean ± SEM g/100g FAME.

Detailed Description of the Drawings

Materials and Methods

Severe Combined Immuno Deficient (SCID) mice were used in this study, which had been induced with colitis through adoptive transfer of splenic CD4⁺ CD45RB^{high} T cells from BALB/c mice. A group of mice (n=8) were fed a linoleic acid (LA) supplemented diet (1%), a second group received pure *cis*-9, *trans*-11 CLA (1%), a third group received LA (1%) together with the CLA-producing strain *B. breve* NCIMB 702258 (a daily dose of 10⁹ organisms), a fourth group were fed a standard diet together with *B. breve* NCIMB 702258 (a daily dose of 10⁹ organisms), and a fifth group were fed only the standard diet. Ten weeks after colitis induction, cytokine production by splenocytes was measured in vitro by ELISA, myeloperoxidase (MPO) activity was determined in colonic homogenates and fatty acid composition in liver, adipose tissue, cecum and colon was determined by gas liquid chromatography (GLC). Similar studies

have also been conducted in pigs (data not shown here) and similar results obtained.

Animals and Experimental design

SCID mice were purchased from Harlan ltd. (Briester, Oxon, UK) at 6 weeks of age and fed a normal diet for 1 week to stabilize all metabolic conditions. Each cage contained one mouse. Mice were exposed to a 12-h light:dark cycle and maintained at a constant temperature of 25°C. One week after arrival, mice were divided into five groups for different dietary treatments (Table I).

For linoleic acid and *c9t11* CLA treatment, a powdered diet blended with the drug was administered for 10 weeks to yield a dose of drug at approximately 90mg/day/mouse (this is based on preliminary experiments by Bassaganya-Riera *et al.* (22) that established an optimal dose of fatty acids of 1g/100g of diet).

Male BALB/c mice were purchased from Harlan Ltd. (Briester, Oxon, UK). One week after arrival, these mice were divided into two groups (n=9) for different dietary treatments. Group A received 1% LA in their diet together with approximately 1×10^9 live *B. breve* NCIMB 702258 per day. Group B received 1% LA in their diet. For LA treatment, a powdered diet blended with the drug was administered for 8 weeks to yield a dose of drug at approximately 90mg/day/mouse (this is based on preliminary experiments by Bassaganya-Riera *et al.* (1) that established an optimal dose of fatty acids of 1g/100g).

Animals were fed standard mouse chow ad libitum with free access to water at all times. The ingredients and composition of the basal diet was as follows:

Ingredients; Soyabean extracted toasted, Cane molasses, Sunflower seed

extracted, Wheat, Barley, Mineral/vitamin, Soya(bean) hulls, Maize gluten, Calcium carbonate (limestone flour), Sodium chloride.

Composition;

Protein 17.0%

Oil 3.0% (background oil, no oil is added. 1.6% comes from wheatfeed, 0.9% from oats, 0.2% from sunflower)

Fibre 8.50%

Ash 7.80%

Moisture 14.0%

Copper 30.00 mg/kg

Vitamin A 13500 iu/kg

Vitamin D 3000 iu/kg

Vitamin E 90 iu/kg

- 5 Of the total fatty acids that are in the diet, Linoleic acid is responsible for approximately 50% (Oleic acid 22%, palmitic acid 17.5%, linolenic acid 3.7% and stearic acid 2.8%). The amount of oil in their diet is 3%, which comes from wheatfeed, soyabean, oats and sunflower.

After 8 weeks on experimental diets, the mice were sacrificed by cervical
10 dislocation. Livers, hearts, colons, small intestines and cecums were removed from the carcasses, blotted dry on filter paper, weighed and frozen in liquid nitrogen. All samples were stored at -80°C until processed.

Induction of colitis

- SCID mice were induced with colitis through adoptive transfer of the CD4⁺
15 CD45RB^{high} subpopulation of CD4⁺ T cells from normal BALB/c mice (Mackay *et al.* 1998).

Probiotic strain

- B. breve* National Culture for Industrial and Marine Bacteria deposit no. 702258 was originally isolated from an infant intestine and has previously been shown to
20 be resistant to intestinal acid and bile and to adhere to human intestinal cells (unpublished data). This strain has previously been shown to be an efficient CLA producer, converting up to 65% LA to *c9, t11* CLA when grown in 0.55 mg ml⁻¹ LA in vitro (23).

- A spontaneous rifampicin resistant variant of the strain was isolated, prior to
25 initiation of this study, in order to facilitate uncomplicated identification of this bacterium from all other bifidobacteria.

Preparation and administration of the probiotic

- B. breve* NCIMB 702258 was initially grown in modified MRS medium (mMRS) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (98% pure; Sigma
30 Chemical Co., St. Louis, Mo.) by incubating overnight at 37°C under anaerobic conditions. The culture was pelleted by centrifugation (7000g, 15 min, 4°C). The pellet was washed twice in PBS (Sigma) and then resuspended at 1x10¹⁰ cells/ml in 15% trehalose (Sigma). 1 ml was aliquoted into 2-ml vials and freeze-dried

using a 24 hour programme (freeze temp -40°C, condenser set point -60, vacuum set point 600 m Torr). Each mouse consumed approximately 1×10^9 live microorganisms per day. This was achieved by resuspending appropriate quantities of freeze-dried powder in the water that the mice consumed *ad libitum*.

5

Assessment of colitis

After the adoptive transfer, mice were weighed three times a week and examined for clinical signs of disease associated with colitis (ie. rectal bleeding, diarrhoea and rectal prolapse). After 10 weeks on experimental diets, the mice were sacrificed by cervical dislocation. Livers, adipose tissue, spleens, colons and cecums were removed from the carcasses, blotted dry on filter paper, weighed and frozen in liquid nitrogen. All samples were stored at -80°C until processed. The colon was divided into different sections for MPO activity and fatty acid composition.

15

Myeloperoxidase (MPO) assay

Myeloperoxidase activity was measured according to the method by Krawisz *et al.* (24). Samples were excised from each animal and rapidly rinsed with ice-cold PBS, blotted dry and frozen at -80°C. The tissue was thawed and homogenized in 0.5 ml PBS. 200 µl of each homogenised sample was transferred to separate eppendorf tubes and 400 µl of 0.5% hexadecyltrimethylammonium bromide (HTAB) solution (Sigma, St Louis, Missouri, USA) was added. After vortexing for 2 min, samples were spun at 5.000 rpm for 5 min after which the supernatant was collected. Using a 96 well microtitre plate, 50 µl of each sample was added to duplicate wells. 12.5 µl of hydrogen peroxide (H₂O₂, 30% (w/v), Sigma) was added to each well followed by 200 µl of O-dianisidine reaction solution (Sigma). The absorbance (OD) was measured spectrophotometrically at 450 nm at 1 min intervals for 15 min. The total soluble protein concentration in the samples was estimated using the method by Bradford *et al.* (48). This was performed using a Bio-Rad protein assay dye reagent kit (Bio-Rad Laboratories, Hercules, CA). Results are expressed as U/mg protein.

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Cytokine production by splenocytes.

Cytokine production in response to defined stimuli, *in vitro*, was measured using enzyme-linked immunosorbent assay (ELISA). Splenocyte isolation was performed using an erythrocyte lysing kit (R&D Systems). The spleens of all mice were removed at the time of sacrifice, blotted on filter paper and weighed.

- 5 Each spleen was immediately placed in Hanks' buffer containing 10% fetal calf serum. The spleen was teased apart and sieved through a cell strainer into a 50 ml centrifuge tube. Cells were centrifuged for 10 minutes at 200 g. The cells were resuspended in 2 ml M-lyse buffer, which lyses red blood cells, and incubated for 8 minutes at room temperature. Lysis was deactivated using wash buffer and the
- 10 cell suspension was centrifuged for 10 minutes at 200 g. Cells were resuspended in DMEM (10% fetal calf serum, 1% Pen/Strep, Sigma) and diluted to 1×10^6 cells/ml for *in vitro* culturing. The isolated lymphocytes were cocultured with the proinflammatory bacterium *Salmonella typhimurium* UK1 (1×10^6 cells/ml) and with antiCD3-antiCD28 monoclonal antibodies for 48 hours at 37°C. Cell
- 15 supernatants were isolated and stored at -80°C. Cytokine analysis was performed on the supernatants using a BDTM Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, US).

Microbial analysis

- Fresh faecal samples were taken directly from the anus of every mouse every
- 20 second week for microbial analysis and fatty acid analysis. Microbial analysis of the samples involved enumeration of *B. breve* NCIMB 702258. This analysis was performed by pour plating onto MRS agar supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (98% pure; Sigma Chemical Co., St. Louis, Missouri, USA), 100 µg of Mupirocin (Oxoid)/ml (4) and rifampicin (Sigma).
- 25 Large intestinal contents were also sampled at sacrifice for enumeration of the administered *B. breve* strain and for enumeration on *Lactobacillus* selective agar (LBS) (Becton Dickinson Co, Cockeysville, USA). Microbial analysis of *B. breve* NCIMB 702258 was performed by pour plating onto mMRS agar supplemented with 100 µg of mupirocin (Oxoid)/ml (Rada, 1997) and 100 µg
- 30 rifampicin (Sigma)/ml. Agar plates were incubated anaerobically for 72 hrs at 37°C. Anaerobic environments were created using CO₂ generating kits (Anaerocult A; Merck, Darmstadt, Germany) in sealed gas jars.

Lipid extraction and fatty acid analysis

Lipids were extracted with Chloroform:Methanol 2:1 v/v according to Folch *et al.* (25). Briefly, tissue or faecal samples, ~1g liver, 300mg small intestine, 200mg adipose tissue, 250mg colon, 800mg cecum or 100mg faeces were
5 homogenised in over a 25 fold excess of CHCl₃:CH₃OH (2:1 v/v) and washed with 0.88% KCl solution. Excess solvent was dried down under a gentle stream of N₂ at 40°C and lipids were stored at -20°C in 1ml chloroform. Fatty acid methyl esters (FAME) were prepared using first 10ml 0.5N NaOH in methanol for 10 min at 90°C followed by 10ml 14% BF₃ in methanol for 10 min at 90°C
10 (26). FAME was recovered with hexane. Prior to GC analysis samples were dried over 0.5g of anhydrous sodium sulphate for an hour and stored at -20°C. FAME were separated by gas liquid chromatography (GLC Varian 3400, Varian, Walnut Creek, CA. USA fitted with a flame ionization detector) using a Chrompack CP Sil 88 column (Chrompack, Middleton, The Netherlands, 100 m
15 x 0.25 mm i.d., 0.20 µm film thickness) and He as a carrier gas. The column oven was programmed to be held initially at 80°C for 8 minutes then increased 8.5°C/min to a final column temperature of 200°C. The injection volume used was 0.6µl with automatic sample injection with a splitless on SPI on-column temperature programmable injector. Data was recorded and analysed on a
20 Minichrom PC system (VG Data System, Manchester, U.K.). Tri-heptadecanoate (Sigma) was used as an internal standard, and peaks were identified with reference to retention times of fatty acids in a standard mixture. All fatty acid results are shown as mean ± SD (n = 8) g /100 g FAME.

Statistical analysis

25 Data are expressed as the mean value per group of mice ± standard deviation. Data was analysed by MINITAB[®] Release 14 statistical software, Lead Technologies, Inc. and data were tested as appropriate by ANOVA or Kruskal-Wallis (27) tests in order to assess if differences between groups are significant. A P-value of < 0.05 was considered to be statistically significant.

30 Preparation and administration of *B. breve* NCIMB 702258

Rifampicin resistant variants of the *B. breve* strain were isolated by spread-plating ~10⁹ colony forming units (CFU) from an overnight culture onto MRS agar (de Man, Rogosa & Sharpe) (Difco Laboratories, Detroit, MI, USA)

supplemented with 0.05% (w/v) L-cysteine hydrochloride (98% pure; Sigma Chemical Co., St. Louis, MO) (mMRS) containing 500 µg/ml rifampicin (Sigma Chemical Co., Poole, Dorset, UK). Following anaerobic incubation at 37°C for 3 days, colonies were stocked in mMRS broth containing 40% (v/v) glycerol and
5 stored at -80°C. To confirm that the rifampicin resistant variant was identical to the parent strain, molecular fingerprinting using pulse-field gel electrophoresis (PFGE) was employed. Both parent and variant strains were routinely cultured at 37°C in mMRS broth in anaerobic jars with CO₂-generating kits (Anaerocult A; Merck, Darmstadt, Germany). Importantly, the rifampicin resistant variant
10 was comparable to the parent strain for CLA production.

Mice that did not receive the bacterial strain received placebo freeze-dried powder (15% w/v trehalose).

Animals and treatment

Female BALB/c mice were purchased from Harlan Ltd. (Briester, Oxon, UK) at 8
15 weeks of age and fed a normal diet for 1 week to stabilize all metabolic conditions. The basal diet contained the following nutrient composition (w/w): 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: C12:0 (0.03%), C14:0 (0.14%), C16:0 (0.33%), C18:0 (0.06%),
20 monounsaturated fatty acids: C14:1 (0.02%), C16:1 (10%), C18:1 (0.87%), polyunsaturated fatty acids: C18:2 n -6 (0.96%), C18:3 n -3 (0.11%), C20:4 n -6 (0.11%). Mice were maintained at 4 per cage and exposed to a 12-h light:dark cycle at a constant temperature of 25°C. The mice were held at the Biological Services Unit in University College Cork. The animal experimentation was
25 performed according to the guidelines for the care and use of laboratory animals approved by the Department of Health and Children.

One week after arrival, mice were divided into four groups (n=8/group) and subjected to the following dietary treatments: Group A were supplemented with 1% α -linolenic acid (w/w, triglyceride bound form, Larodan Fine Chemicals AB,
30 Malmo, Sweden) in combination with approximately 1×10^9 live *B. breve* NCIMB 702258 per mouse/day. Group B were supplemented with 1% α -linolenic acid and placebo freeze-dried powder, group C received standard diet supplemented with $\sim 1 \times 10^9$ live *B. breve* NCIMB 702258, and group D received

standard diet and placebo freeze-dried powder. Animals were fed standard mouse chow *ad libitum* with free access to water at all times. For α -linolenic acid treatment, a powdered standard diet was blended with the α -linolenic acid to yield a concentration of approximately 90 mg α -linolenic acid/day/mouse (based on Bassaganya-Riera *et al.* (19) who reported an optimal intake of fatty acids of 1g/100g). Following 8 weeks on experimental diets, the animals were sacrificed by cervical dislocation. Liver, adipose tissue and brain were removed from the carcasses, blotted dry on filter paper, weighed and frozen in liquid nitrogen. All samples were stored at -80°C until processed.

10 **Microbial analysis**

Lipid extraction and fatty acid analysis

Lipids were extracted according to the method by O'Fallon *et al.* (20). Briefly, samples were cut into 1.5-mm rectangular strips and placed into a screw-cap Pyrex culture tube together with 0.7 ml of 10 N KOH in water and 5.3 ml of MeOH. The tubes were incubated in a 55°C water bath for 1.5 h with vigorous hand-shaking every 20 min. After cooling below room temperature, 0.58 ml of 24 N H₂SO₄ in water was added. The tubes were mixed by inversion and with precipitated K₂SO₄ present incubated again in 55°C for 1.5 h with hand-shaking every 20 min. FAME were recovered by addition of 3 ml hexane and vortex mixed and separated by GLC (Varian 3400, Varian, Walnut Creek, CA. USA fitted with a flame ionization detector) using a Chrompack CP Sil 88 column (Chrompack, Middleton, The Netherlands, 100m×0.25mm i.d., 0.20 μ m film thickness) and He as carrier gas. The column oven was initially programmed at 80°C for 8 min, and increased 8.5°C/min to a final column temperature of 200°C. The injection volume was 0.6 μ l, 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 System, Manchester, U.K.). Peaks were identified with reference to retention times of fatty acids in a standard mixture. All fatty acid results are shown as mean $\pm$ standard error mean (SEM) g /100 g FAME.

Results

In this study, the ability of probiotic bacteria to exert anti-inflammatory effects through the production of CLA was assessed. The conversion of LA to *c9, t11* CLA by *B. breve* NCIMB 702258 was investigated in vivo and also whether this
5 bacterially produced CLA would have any beneficial effect on Inflammatory Bowel Disease (IBD). Throughout the study period, faecal recovery of *B. breve* NCIMB 702258 was confirmed in all probiotic fed mice but not in controls.

10 Myeloperoxidase (MPO) assay

Myeloperoxidase (MPO) activity, an index of quantitative inflammation and neutrophil infiltration in the mucosa was measured in colonic homogenates. As shown in Table II, the MPO activities were much higher in the placebo fed group than in other groups. The MPO activity was lowest in the mice fed *c9, t11* CLA
15 although this was not significantly different from other groups. The MPO activity proved to be highly variable between animals within the same group.

Cytokine production

Cytokine analysis was performed on splenocyte supernatants by ELISA following stimulation in vitro with the proinflammatory bacterium *S. typhimurium* UK1 and with antiCD3-antiCD28 monoclonal antibodies. The
20 proinflammatory cytokines interferon- γ (IFN- γ), tumour necrosis factor α (TNF- α), and interleukin-6 (IL-6) were significantly reduced in the mice fed *c9, t11* CLA (group 4) following stimulation with antiCD3-antiCD28 compared to placebo mice (Fig 1, A-C). IFN- γ levels in the control group were 3093.4
25 compared with 1334.2 in the *c9, t11* CLA group ($p < 0.01$). TNF- α levels in the control group were 838.5 compared with 458.9 in the *c9, t11* CLA group ($p < 0.05$). Furthermore, the IL-6 levels in the control group were 528.4 compared with 213.7 in the *c9, t11* CLA group ($p < 0.05$). Moreover, TNF- α levels following stimulation with *S. typhimurium* UK1 showed a significant reduction
30 in the *c9, t11* CLA fed group. TNF- α levels in the control group were 2418.8 compared with 1428.7 in the *c9, t11* CLA group ($p < 0.05$) (Fig 1E).

IFN- γ and IL-6 were also reduced in this group given *c9, t11* CLA following stimulation with *S. typhimurium* UK1, although these did not reach significant differences (Fig 1, D and F).

TNF- α levels following stimulation with antiCD3-antiCD28 showed a
5 significant reduction in the probiotic group (group 3). TNF- α levels in the control group were 838.5 compared with 414.1 in the *B. breve* NCIMB 702258 group ($p < 0.05$) (Fig 1B). In addition, TNF- α and IFN- γ were significantly reduced in the mice fed *B. breve* NCIMB 702258 following stimulation with *S. typhimurium* UK1 (Fig 1, D-E). TNF- α levels in the control group were 2418.8
10 compared with 839.0 in the *B. breve* NCIMB 702258 group ($p < 0.05$). IFN- γ levels in the control group were 145.2 compared with 75.7 in the *B. breve* NCIMB 702258 group ($p < 0.05$). Moreover, this group given *B. breve* NCIMB 702258 showed a significant reduction in IL-12 p70 ($p < 0.05$). The mice receiving the probiotic had also a reduction in IL-6, although it did not reach
15 significant difference. There was no reduction in proinflammatory cytokines in the mice fed either LA (group 1) or LA together with *B. breve* NCIMB 702258 (group 2) compared to control.

Tissue fatty acid composition

As expected, the mice that were fed pure *c9, t11* CLA had a much higher amount
20 of *c9, t11* CLA in all tissues ($P < 0.001$). This group had also a decrease in arachidonic acid, 20:4n-6 (AA) in the liver when compared to other groups (Table III) and a significant decrease in 20:4n-6 in cecum digests ($P < 0.05$) (Table IV). The mice within this group had furthermore a significantly higher level of 16:0 (palmitic acid) in the colon and higher levels of 16:1n-7
25 (palmitoleic acid) in all tissues (data not shown).

The group that was fed LA together with *B. breve* NCIMB 702258 (group 2), showed an overall higher amount of *c9, t11* CLA in all tissues when compared to other groups (excluding the *c9, t11* CLA group) (Table V). This group had, similar to the *c9, t11* CLA fed group, a significant reduction of 20:4n-6 in cecum
30 digests. Compared to the LA-fed group this group had a lower amount of LA (18:2n-6) in the liver (Table III). Furthermore, compared to the control group this group had a significant decrease in 22:6n-3 (DHA) in cecum digests and colon.

The mice given *B. breve* NCIMB 702258 (group 3) showed a significantly 3-fold higher amount of the beneficial fatty acid 20:5n-3 (EPA) in adipose tissue compared to control (0.45 ± 0.11 compared with 0.15 ± 0.09 g/100g FAME).

They had an approximately 1.5-fold higher amount of EPA in liver, cecum and colon, although these did not reach significant differences. This group had also a significantly higher amount of 22:6n-3 in adipose tissue. In addition, the AA/EPA ratio was lower in the *B. breve* NCIMB 702258 fed group compared to other groups (Table III and IV). Overall the fatty acid composition was highly variable between animals within the same group.

Effect of the administration of *B. breve* NCIMB 702258 on the amounts of *c9t11*-CLA in different tissues.

When a diet of 1% LA (w/w) was fed, the amounts of *c9, t11* CLA in liver, colon and cecum digests harvested 10 weeks after the initiation of *B. breve* NCIMB 702258 were 4.0-, 3.0- and 2.0-fold higher, respectively, in the mice that received *B. breve* NCIMB 702258 compared to other groups (Table V). In addition, these mice were the only mice that had *c9, t11* CLA incorporated into adipose tissue. They had also a 2-fold increase of *c9, t11* CLA in their faeces.

Furthermore, in comparison with group 1 that only received LA, this group had a lower amount of LA in their livers (Table III), indicating that LA has been metabolized. Altogether, these results indicate that the LA-conjugation activity in the intestine is due to the administered *B. breve* NCIMB 702258 and furthermore it confirms the in vivo incorporation of *c9, t11* CLA into different tissues.

Microbial analysis

Faecal samples were analyzed to assess transit of the probiotic strain. *B. breve* NCIMB 702258 was recovered in faeces from all mice in group A, within 2 weeks of feeding, confirming survival and transit of the probiotic in the mice. Stool recovery of *B. breve* NCIMB 702258 was approximately 1×10^6 CFU/g faeces by week 8 of feeding (Fig. 1). The probiotic strain was not isolated from any of the mice in group B.

BALB/c mouse feeding trial

Body weight

Figure 2 shows mean body weight development during the experimental period. Body weight increased from 25.3g to about 27.6g in group A and from 25.9g to about 29.4g in group B. No significant difference was observed between experimental groups.

5 **Effect of the administration of *B. breve* NCIMB 702258 on the amounts of *c9,t11*-CLA in different tissues.**

The *c9, t11* CLA composition of the livers, colons, small intestines and cecum digests are shown in Fig. 3.

After 8 weeks of feeding there was a significant 2-fold increase of *c9, t11* CLA
 10 in the livers of the mice that were fed LA together with *B. breve* NCIMB 702258, compared to placebo that were fed only LA (0.12 ± 0.06 g/100 g FAME compared to 0.05 ± 0.03 g/100g FAME, $p=0.009$). This oral administration of *B. breve* NCIMB 702258 also resulted in a significant 2-fold increase of *c9, t11* CLA in the large intestine of group A (0.15 ± 0.05 g/100 g FAME compared to
 15 0.07 ± 0.02 g/100 g FAME, $p=0.001$). Significantly higher amounts of *c9, t11* CLA was also observed in the small intestine of the mice receiving *B. breve* NCIMB 702258 (0.05 ± 0.01 compared to 0.03 ± 0.01 , $p=0.04$). Furthermore, higher levels of *c9, t11* CLA were found in the cecum digesta of the mice within group A compared to group B, although these did not reach significant
 20 differences. The mice within group A had also a 2.4-fold significantly higher amount of *c9, t11* CLA in their faeces harvested after 8 weeks compared to group B (0.83 ± 0.25 g/100 g FAME compared to 0.35 ± 0.25 g/100 g FAME, $p=0.001$) (Fig. 4). This higher amount of *c9, t11* CLA correlated with a significantly lower amount of LA in the faeces (15.7 ± 3.80 in group A compared to 27.4 ± 7.16 in
 25 group B, $p=0.001$), with the correlation coefficient (r) being -0.863 (Fig. 4). In addition, in comparison with group B that only received LA, group A had a lower amount of LA in the liver, colon, small intestine and cecum digesta, indicating that LA has been metabolized (Table II). *c9, t11* CLA was not detected in the heart of any of the mice within group A.

30 Altogether, these results prove that administration of *B. breve* NCIMB 702258 to mice increases CLA production in the large intestine, which results in increased CLA absorption and *in vivo* incorporation of *c9, t11* CLA into the liver.

Tissue fatty acid composition

The fatty acid composition of the livers, colons, small intestines and cecum digestas are presented in Table II.

Apart from an overall higher level of *c9*, *t11* CLA and a lower level of LA in group A compared to group B, there was also a significantly higher level of stearic acid (18:0) in the colon of this group ($p=0.02$) and an overall lower level of oleic acid (18:1 $c9$) in this group. Furthermore, the mice within this group had a significantly higher amount of eicosapentaenoic acid (EPA, 20:5 $n-3$, $p=0.041$) and docosahexaenoic acid (DHA, 22:6 $n-3$, $p=0.008$) in the colon, and also a significantly higher level of dihomo- γ -linolenic acid (20:3 $n-6$, $p=0.027$). EPA and DHA were also overall higher in the group administered *B. breve* NCIMB 702258. In addition, the delta-6 desaturation index $[(18:3n-6 + 20:3n-6)/18:2n-6]$ in the colon was significantly higher in the mice receiving *B. breve* NCIMB 702258 compared to the placebo group (0.018 ± 0.004 compared to 0.014 ± 0.005 , $p=0.045$).

15 Analysis of other CLA-producing strains

Other strains were identified which produce CLA, as follows;

Strain	% conversion to <i>c9</i> , <i>t11</i> CLA	% conversion to CALA
<i>B. breve</i> DPC 6330	76.65 ± 1.75	~ 71
<i>B. breve</i> DPC 6331	61.12 ± 3.85	~ 7
<i>B. breve</i> NCIMB 702258	60%	45%
<i>B. longum</i> DPC 6315	60.12 ± 5.14	none producer
<i>B. longum</i> DPC 6320	53.08 ± 2.51	none producer

Table VI CLA-producing strains.

20

Animal performance

Mice were weighed twice a week over the 8 week trial period. Oral administration of *B. breve* NCIMB 702258 and/or α -linolenic acid did not significantly influence body weight gain throughout the trial period.

25

Microbial analysis

The numbers of *B. breve* NCIMB 702258 were monitored in the faeces of individual mice every 14 days. The administered *B. breve* was recovered in faeces from all mice that received the strain, within 2 weeks of feeding,

confirming gastrointestinal transit and survival of *B. breve* NCIMB 702258. Stool recovery of *B. breve* NCIMB 702258 was approximately 4×10^5 CFU/g faeces by week 8 of the trial in mice that received *B. breve* in combination with α -linolenic acid (group A) and approximately 2.2×10^6 CFU/g faeces in mice that received *B. breve* without α -linolenic acid (group C) ($p > 0.05$). The *B. breve* strain was detected in large intestinal contents at $\sim 4.6 \times 10^5$ CFU/g in mice that received *B. breve* and α -linolenic acid (group A) and $\sim 1.4 \times 10^6$ CFU/g in mice that received *B. breve* alone (group C) at sacrifice ($p > 0.05$) (Fig. 6). *B. breve* NCIMB 702258 was not isolated from any of the mice within group B (administered α -linolenic acid alone) or group D (placebo control). Following culturing of the large intestinal content on *Lactobacillus* selective media (LBS), the numbers of CFU obtained did not differ significantly between the groups ($p > 0.05$) (Fig. 6).

Tissue fatty acid composition

Administration of *B. breve* in combination with α -linolenic acid resulted in significant changes in the fatty acid composition of host tissues including liver and brain compared to animals administered α -linolenic acid alone. Mice that received *B. breve* in combination with α -linolenic acid (group A) exhibited 23% higher EPA and 20% higher dihomogamma-linolenic acid (C20:3n-6) in liver compared with the group administered α -linolenic acid alone (group B, $p < 0.05$, Fig 7, Table I). The former group also exhibited a 12% higher DHA concentration in brain ($p < 0.05$, Fig 8) as well as numerically higher concentrations of DHA in adipose tissue and liver (27% and 16%, respectively) (Table II). Furthermore, this group exhibited significantly decreased n-6/n-3 ratio in brain tissue compared with the latter group ($p < 0.05$) (Table II).

Oral administration of *B. breve*, both in combination with α -linolenic acid and without α -linolenic acid supplementation (group A and C), resulted in significantly higher ($p < 0.05$) concentrations of arachidonic acid (C20:4n-6) and stearic acid (C18:0) incorporated in liver compared with mice that did not receive the bacterial strain (group B and group D) (Table I). Moreover, the mice that received *B. breve* supplementation, without α -linolenic acid (group C), exhibited numerically higher concentration (16%) of DHA in brain tissue compared with

unsupplemented controls (group D), although this was not statistically significant (Table II).

Supplementation of α -linolenic acid, either in combination with *B. breve* or in the absence of the *B. breve* strain (group A and group B) resulted in 10-fold
5 higher concentrations of α -linolenic acid and EPA in liver and adipose tissue compared with groups that did not receive the fatty acid supplement (group C and group D) ($p < 0.001$) (Table I). In addition, these groups also exhibited significantly higher concentrations of docosapentaenoic acid (DPA, C22:5 n -3) in liver and adipose tissue ($p < 0.05$), significantly higher concentrations of DHA in
10 liver ($p < 0.05$) and significantly lower levels of arachidonic acid in liver, adipose tissue and brain ($p < 0.05$) compared with groups that did not receive α -linolenic acid supplementation (group C and group D) (Table I and II). The arachidonic acid/EPA ratios in liver and adipose tissue were approximately 30-fold and 20-fold lower, respectively, in the groups that received α -linolenic acid (group A
15 and B) compared with animals that did not receive the fatty acid (group D). In addition, the n -6/ n -3 ratios were significantly lower in all tissues, except the brain of animals supplemented with α -linolenic acid (group A and B) ($p < 0.001$). Supplementation with α -linolenic acid also resulted in significant decreases in concentrations of palmitic acid (C16:0), palmitoleic acid (C16:1 n -7) and oleic
20 acid (C18:1 n -7) ($p < 0.001$), and significantly higher stearic acid (C18:0) in liver compared with unsupplemented groups ($p < 0.001$) (Table I).

Discussion

This study sought to investigate whether administration of a CLA-producing strain would produce CLA *in vivo* and furthermore whether this bacterial
5 produced CLA would ameliorate colonic inflammation. Although, only limited data on the effects of probiotics on dietary fatty acids has been published, there are indications that intestinal bacteria may interact with different fatty acids. To our knowledge, this is a first-time observation where the effects of oral intake of CLA-producing bacteria on inflammatory bowel disease have been investigated.

10 Certain metabolic capabilities of microorganisms that easily are observed *in vivo* do not necessarily occur *in vitro*. However, results from this study prove that linoleic acid has been converted to c9t11-CLA by *B. breve* NCIMB 702258 *in vivo* and furthermore it confirms the *in vivo* incorporation of c9t11-CLA into different tissues. Administration of *B. breve* NCIMB 702258 to mice increased
15 the CLA production in the large intestine threefold and the CLA content of the liver fourfold. Altogether, this proves that administration of *B. breve* NCIMB 702258 to mice increases CLA production in the large intestine which results in increased CLA absorption. This is in agreement with a study made by Fukuda *et al.* (30), who administered a CLA-producing strain, *Butyrivibrio fibrisolvens*
20 MDT-5, every other day to mice for two weeks. This oral administration resulted in increased amounts of CLA in the contents of the large intestine (2.5-fold) as well as in adipose tissue (threefold). Feeding a high-LA diet, as well as prolonging the period of MDT-5 administration, further increased the CLA content in body fat. The MDT-5 strain is a bovine bacterium and not a human
25 strain, like those of the invention, so it is not part of the human gut microflora. Fukuda *et al* did not show production of CLA in the liver or any effects on inflammation. A subsequent study in rats concluded that gut microbes did not lead to increased CLA production *in vivo*. In contrast, a study made by Kamlage *et al.* (31) concluded that intestinal microorganisms do not supply rats with
30 systemic CLA, insofar as CLA did not accumulate in tissues upon administration of LA conjugating microbes to germ-free rats. They found however that LA-conjugation activity in feces was increased after administrating microbes known to produce CLA. They concluded that this may be due to the inability of the microorganism investigated to conjugate LA *in vivo*. However, the strain used in

this study, *B. breve* NCIMB 702258, proved to conjugate LA into CLA in SCID mice in vivo. Little is known about the intestinal absorption of CLA. However, the intestines are the first step of nutrient delivery to tissues and, as such, may modulate the bioavailability of ingested fatty acids and also, therefore, their biological effects. The absorption of *c*9, *t*11 CLA and *t*10, *c*12 CLA in the small intestine is particularly unclear (49). Since LA has proinflammatory capabilities and has furthermore been reported to act as a promoter of carcinogenesis, conversion of LA to CLA by bacteria in the intestine may be important to reduce LA absorption following ingestion of high-LA acid diets. In the typical western diet, 20-25 fold more n-6 fats than n-3 fats are consumed. This predominance of n-6 fat is due to the abundance in the diet of LA, which is present in high concentrations in soy, corn, safflower, and sunflower oils. LA is converted to arachidonic acid (AA) and AA is the precursor for the proinflammatory eicosanoids prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄), which are maintained at high cellular concentrations by the high n-6 and low n-3 polyunsaturated fatty acid content of the modern western diet. Furthermore, in order to maintain health, CLA needs to be taken continually, but large doses of CLA as a supplement may have deleterious effect. Therefore, it is desirable to absorb CLA produced slowly and continually in the large intestine. This study shows that *B. breve* NCIMB 702258 may be useful as a probiotic to provide CLA in the large intestine continuously.

Although CLA was produced in the group fed *B. breve* NCIMB 702258 and LA, these mice did not show any amelioration of the proinflammatory cytokines IL-6, IFN- γ and TNF- α . This is probably due to the high amount of LA fed. As mentioned above, LA has been shown to have proatherogenic and proinflammatory properties. For example LA has been shown to increase the activation of NF- κ B and expression of cytokines and cell adhesion molecules .

Consistent with previous observations in liver of mice (35), the analysis of the fatty acid composition of liver in this study revealed that the dietary CLA supplementation to one group of mice decreased the concentration of arachidonic acid (AA). The mice fed *c*9, *t*11 CLA had a significantly lower amount of AA in the cecum and furthermore a lower amount incorporated in their livers. AA is a precursor for the generation of first-phase eicosanoids (i.e., two series

prostaglandins and four series leukotrienes) involved in early microinflammatory events (i.e., polymorpho-nuclear neutrophilic leukocyte chemotaxis and release of superoxide anions). Enhanced intestinal eicosanoid concentrations closely correlate with severe signs of colonic inflammation. Results from this study show that dietary intake of *c9, t11* CLA inhibits hepatic and intestinal 20:4(n-6) synthesis. This may in turn down-regulate the production of pro-inflammatory eicosanoids. The beneficial impact of *c9, t11* CLA on the mucosa was associated with changes in the systemic cytokine production in vitro. Following challenge (either with antiCD3-antiCD28 monoclonal antibodies or *S. typhimurium* UK1), there was a reduction in the Th1 cytokines TNF- α and IFN- γ and in the Th2 cytokine IL-6 in the mice fed this fatty acid. Research in several species, including poultry (38), rats (39), and mice (40), have shown that dietary CLA can reduce the release of proinflammatory cytokines. At the molecular level, the concentration of cytokines in tissues is controlled in part by mechanism(s) of transcriptional regulation. CLA works as an activator for PPAR- γ . PPAR- γ activation has been demonstrated to antagonize the activities of several transcription factors including NF- κ B. As a result of this interference with the NF- κ B signalling pathway, the expression of proinflammatory cytokines (i.e., TNF- α , IL-6 and IL-1 β) is suppressed and macrophage apoptosis induced, both effects with likely consequences in inflammation.

In addition, the activity of MPO (an index of tissue-associated neutrophil accumulation) was also lower in the mice fed *c9, t11* CLA.

Although we did not notice any alleviation of the proinflammatory cytokines in the mice fed *B. breve* NCIMB 702258 and LA (probably due to the high amount of LA fed), we noticed a positive effect on the mucosal inflammation in the group fed only *B. breve* NCIMB 702258. This probiotic effect was reflected by a reduction in the proinflammatory cytokines IL-6, IFN- γ , IL-12p70 and TNF- α secreted by splenocytes. Alteration in cytokine profiles observed is important as IBD is associated with a predominance of Th1 cytokines (e.g. IFN- γ , IL-12p70 and TNF- α). Previous probiotic trials have shown the same probiotic efficacy in inflammatory disorders as seen in this study. For example, in a study by McCarthy *et al.* (29) both *Lactobacillus salivarius* 433118 and *Bifidobacterium infantis* 35624 attenuated colitis through a significant

amelioration of proinflammatory IFN- γ and TNF- α . These strains however did not produce CLA. Treatment of IL-10 knockout mice with VSL#3, *Lactobacillus plantarum* 299v and *Escherichia coli* ssp. *laves* have demonstrated improvements in inflammation and histological disease in conjunction with significantly decreased mucosal secretions of IFN- γ and TNF- α (45-47). The MPO-activity was also lower in the mice fed *B. breve* NCIMB 702258 signifying the probiotic-mediated immune modulation by this strain.

Interestingly, we noticed an altered PUFA composition in the group given *B. breve* NCIMB 702258 compared to the control group. This alteration included a threefold significantly higher amount of the beneficial fatty acid 20:5n-3 (EPA) in the adipose tissue. The mice within this group had also a higher amount of EPA in liver, cecum and colon, and a significantly higher amount of DHA in adipose tissue. In similar action to CLA, 20:5n-3 also works as an activator of PPARs. For example, EPA- activated PPAR- γ induces lipoprotein lipase and fatty acid transporters and enhances adipocyte differentiation as well as inhibits the function of the transcription factor NF- κ B and cytokines. Furthermore, the AA/EPA ratio was much lower in the *B. breve* NCIMB 702258 fed group compared to other groups. This suggests that *B. breve* NCIMB 702258 modifies the fatty acid composition to a more beneficial composition. Although only limited data on the effects of probiotics on dietary fatty acids has been published, there are indications that intestinal bacteria may interact with different fatty acids. There are at least two possible mechanisms by which *B. breve* NCIMB 702258 could achieve the effects seen in the present study; (i) *B. breve* NCIMB 702258 could influence the fatty acid composition by either utilizing or simply assimilating certain PUFAs, or (ii) *B. breve* NCIMB 702258 could influence the mechanisms of dietary PUFA uptake to the intestinal epithelium. Whether this modulation in fatty acid composition by *B. breve* NCIMB 702258 is the responsible mechanism for the probiotic attenuation of colitis seen here requires further validation.

Proinflammatory cytokine production by splenocytes was significantly reduced in the groups fed the probiotic strain *B. breve* NCIMB 702258 and pure *cis*-9, *trans*-11 CLA. Consumption of *B. breve* NCIMB 702258 resulted in a significant amelioration of the proinflammatory Th1 cytokine tumour necrosis

factor α (TNF- α) and a reduction of the proinflammatory Th1 cytokine interferon- γ (IFN- γ) compared to placebo. Consumption of *c9, t11* CLA lead to a significant reduction in IFN- γ , TNF- α , and interleukin-6 (IL-6).

The group given *B. breve* NCIMB 702258 had a significantly threefold
5 higher amount of the beneficial fatty acid 20:5n-3 (EPA) in adipose tissue compared to placebo. In addition, the arachidonic acid (AA)/eicosapentaenoic acid (EPA) ratio was lower in this group compared to other groups.

In the group fed *B. breve* NCIMB 702258 together with LA, there was a significant increase of *c9t11*-CLA in the liver compared to other groups
10 (excluding the *c9, t11* fed group). This group had also an increase of *c9t11*-CLA in colon (threefold) and was furthermore the only group that had *c9t11*-CLA incorporated into the adipose tissue. Altogether these results prove that LA has been converted to *c9t11*-CLA by *B. breve* NCIMB 702258 *in vivo*. This group didn't show any amelioration in the immune response however which could be
15 due to the high amount of LA fed.

Linoleic acid (LA; *cis*-9, *cis*-12-18:2) is metabolised by bacteria in the rumen of ruminants in a process known as biohydrogenation. This process carries important implications for the fatty acid composition of milk. The first step in the biohydrogenation by the rumen bacteria is the isomerisation of the *cis*-12 double
20 bond of LA to a *trans*-11 configuration resulting in *c9, t11* CLA (*cis*-9, *trans*-11-18:2). Next step is a reduction of the *cis*-9 double bond resulting in a *trans*-11 fatty acid; vaccenic acid (VA; *trans*-11-18:1). Both this fatty acids are considered to be beneficial for health. The final step in the biohydrogenation is a further hydrogenation of the *trans*-11 double bond in VA, producing stearic acid (18:0)
25 as a final product. The microbiology of biohydrogenation in the rumen has received lots of attention, but similar research has not been carried out for the human intestinal microflora. CLA has been shown to exert a variety of beneficial biological activities in several experimental animal models. In this study we investigated whether a *c9, t11*-CLA producing *Bifidobacterium* strain of human
30 origin; *B. breve* NCIMB 702258 could produce the bioactive *c9, t11*-CLA from LA *in vivo*.

This study shows that an eight week oral treatment with the CLA-producing strain *B. breve* NCIMB 702258 increases the content of *c9, t11* CLA

in the colon, small intestine, and liver significantly, proving the *in vivo* c9, t11-CLA production by this strain. This is in agreement with a study made by Fukuda *et al.* (30). , They reported that CLA was produced from LA when human faeces were incubated *in vitro*, but substantially no CLA was produced
5 when the same fecal bacteria were introduced into germ-free rats. They stated that the difference between the *in vivo* and *in vitro* results is explainable by the fact that CLA production is inhibited by glucose (16).

It is now well established that CLA has antiproliferative and anti-inflammatory effects on colonocytes, so the provision of CLA in the intestinal
10 lumen would be considered beneficial, particularly for inflammatory bowel disease, such as ulcerative colitis and Crohn's disease. *B. breve* NCIMB 702258 is expected to continuously produce CLA after it colonises the gut, thereby continuously exerting beneficial effects from CLA. Furthermore, in order to maintain health, CLA needs to be taken continually, but large doses of CLA as a
15 supplement may have deleterious effect. Therefore, it is desirable to supply a low level of CLA frequently or continuously to the body. This study shows that *B. breve* NCIMB 702258 may be useful as a probiotic to provide CLA in the large intestine continuously.

In addition, since LA has proinflammatory capabilities and have
20 furthermore been reported to act as a promoter of carcinogenesis , conversion of LA to CLA by bacteria in the intestine may be important to reduce LA absorption following ingestion of high-LA acid diets. The mice receiving *B. breve* NCIMB 702258 had an overall lower amount of LA in the liver, colon, small intestine and cecum digesta compared to the LA-fed group, indicating that
25 LA has been metabolized by *B. breve* NCIMB 702258.

The amount of LA available for CLA production in the colon is varying and is dependant upon the amount ingested and the efficacy of absorption in the small intestine. Edionwe *et al.* (22) showed that humans generally excrete ~ 20 mg of LA/day, suggesting that substrate is available for microbial production of
30 CLA.

Stearic acid, the final product of LA metabolism by bacteria, was significantly higher in the colon of the mice fed *B. breve* NCIMB 702258. This is in agreement with a study made by Howard *et al.* (21). They showed that bacteria

from the human colon can hydrogenate LA to stearic acid. In their study, human faecal suspensions were incubated with LA for 4 h. As a result of this incubation, LA was significantly decreased and there was a significant rise in its hydrogenation product, stearic acid.

5 We also noticed an altered PUFA composition in the mice given *B. breve* NCIMB 702258 compared to the control group. This alteration included for example significantly higher levels of the beneficial fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the colon. In similar action to CLA, EPA also works as an activator of PPARs. For example, EPA- activated
10 PPAR- γ induces lipoprotein lipase and fatty acid transporters and enhances adipocyte differentiation as well as inhibits the function of the transcription factor NF- κ B and cytokines. The delta-6 desaturation index $[(18:3n-6 + 20:3n-6)/18:2n-6]$ in the colon was also higher in the mice receiving the *Bifidobacterium* strain. Fukushima *et al.* concluded that feeding a probiotic
15 mixture of organisms (*Bacillus*, *Lactobacillus*, *Streptococcus*, *Clostridium*, *Saccharomyces*, and *Candida*) increases the delta-6 desaturase activity in the liver of rats (30).

The influence of dietary PUFA on phospholipids, their eicosanoid derivatives and the transmembrane-signalling lipid rafts into which they are arranged
20 provide multiple mechanisms for dietary modulation of the balance of inflammatory mediators in the human gut. In addition, gut mucosal inflammation is now recognized as being heavily influenced by the composition of the gastrointestinal microbiota (21, 22). Although probiotics and dietary PUFA have been considered separately for the management of inflammation, we propose that
25 these two interventions may act synergistically. To investigate this, we administered *B. breve* NCIMB 702258 in combination with α -linolenic acid to mice in order to assess how this combination would affect the EPA and DHA composition of different host tissues. We found that dietary supplementation with *B. breve* NCIMB 702258 in combination with α -linolenic acid resulted in
30 modulation of host fatty acid composition, and particularly resulted in significantly higher EPA and dihomo- γ -linolenic acid in liver and higher DHA in brain compared to mice that received α -linolenic acid without microbial supplementation. Kaplas *et al.* (8) demonstrated that administration of probiotics

(*L. rhamnosus* GG and *B. animalis* subsp. *lactis* Bb12) to pregnant women resulted in higher concentrations of dihomio- γ -linolenic acid in placental fatty acids. Interestingly, the n -6/ n -3 ratio in brain was also significantly lower in mice that received *B. breve* in combination with α -linolenic acid compared to mice
5 supplemented with α -linolenic acid alone. Moreover, oral administration of *B. breve*, both in combination with α -linolenic acid and without α -linolenic acid supplementation, resulted in significantly higher amounts of arachidonic acid incorporated in liver compared to mice that did not receive *B. breve*. Given that
10 administration of *B. breve* resulted in significantly higher concentrations of long-chain PUFA such as EPA, DHA and also arachidonic acid, administration of this strain resulted in an increase in the levels of unsaturation within fatty acids. Interestingly, it was previously shown that a variety of probiotics increased the activity of liver Δ 6-desaturase in rats, which resulted in increased amounts of arachidonic acid derived from linoleic acid (23).

15 Supplementation of α -linolenic acid both in combination with *B. breve* and in the absence of the *B. breve* strain resulted in significant increases in EPA and DHA in the liver and adipose tissues, at the expense of arachidonic acid. Since EPA replaces arachidonic acid as an eicosanoid substrate in cell membranes of platelets, erythrocytes, neutrophils, monocytes and hepatocytes (24), this results
20 in a reduced synthesis of inflammatory eicosanoids from arachidonic acid and subsequently elevated production of anti-inflammatory eicosanoids from EPA. This alteration towards a more anti-inflammatory profile could be of importance in a variety of chronic inflammatory settings such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease and certain
25 psychiatric diseases such as depression, which are characterized by an excessive production of arachidonic acid-derived eicosanoids (25-27). Moreover, since excessive intake of n -6 PUFA, characteristic of modern Western diets, could potentiate inflammatory processes and so could predispose to or exacerbate associated diseases, increasing the intake of α -linolenic acid and/or EPA may
30 have a protective effect. Supplementation with α -linolenic acid was also associated with a decrease in palmitic acid, palmitoleic acid and oleic acid in liver and adipose tissue, and higher concentrations of stearic acid in these tissues. Similar findings were obtained by Fu and Sinclair (28) who reported that guinea

pigs fed a high α -linolenic acid diet had significantly lower levels of palmitic acid in liver and adipose tissue compared to guinea pigs fed a low α -linolenic acid diet.

Since the effect of combined *B. breve* and α -linolenic acid intervention on EPA-
5 and DHA concentrations was greater than that of α -linolenic acid intervention alone, this effect could be attributed to *B. breve* NCIMB 702258 and thus suggest that feeding a metabolically active strain can influence the fatty acid composition of host tissues. The mechanism by which *B. breve* NCIMB 702258 mediated the changes in host *n*-3 fatty acid composition seen in the present study remains
10 unclear. A possible mechanism may be the properties of bacteria in regulating desaturase activity involved in the metabolism of fatty acids (23).

In conclusion, the present study shows that administration of *B. breve* NCIMB 702258 is associated with alterations in the fatty acid composition of host liver and brain. This study suggests a definite role for the interactions between PUFAs
15 and commensal bacteria. This “synergistic” effect of commensals and fatty acids could be of therapeutic beneficial for a range of immuno-inflammatory disorders as well as having significance for the promotion of neurological development in infants.

Conclusion

20 The presented study opens possibilities to improve the quality of life of IBD patients by using probiotic bacteria. The results suggest that some physiological effects of probiotics e.g. immunomodulating properties, may be associated with physiological interactions between probiotics and polyunsaturated fatty acids (PUFA). Furthermore, this study provides evidence for the *in vivo* CLA
25 production by *B. breve* NCIMB 702258

Thus the introduction of a CLA-producing probiotic strain may enhance the CLA production in the large intestine and could possible contribute to the anti-inflammatory effect and furthermore the prevention of inflammatory bowel disease. The results from this study suggest a definite role for interactions
30 between fatty acids and commensals. Furthermore, that data indicate that the synergist effect between administered microbes and fatty acids could result in more efficient probiotic preparations, and indicate the anti-inflammatory potential of this dietary combination.

The words “comprises/comprising” and the words “having/including” when used herein with reference to the present invention are used to specify the presence of stated features, integers, steps or components but does not preclude
5 the presence or addition of one or more other features, integers, steps, components or groups thereof.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the
10 invention which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination.

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Table I. Dietary treatments of different groups (n = 8).

Group 1	1% LA in standard diet
Group 2	1% LA in standard diet and <i>B. breve</i> NCIMB 702258 (a daily dose of 10^9 organisms)
Group 3	Standard diet and <i>B. breve</i> NCIMB 702258 (a daily dose of 10^9 organisms)
Group 4	1% <i>c9, t11</i> CLA in standard diet
Group 5	Standard diet

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Table II. Colonic mucosal Myeloperoxidase activity (MPO, U/mg protein).

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Group	MPO colon (U/mg protein)
1 (LA)	1.16±0.23
2 (LA + probiotic)	0.98±0.57
3 (probiotic)	0.95±0.86
4 (<i>c9, t11</i> CLA)	0.86±0.73
5 (placebo)	2.18±1.51

Data are expressed as mean ± SD (n = 8).

Table III. Bioproduction of *c*9, *t*11 CLA by *B. breve* NCIMB 702258 occurs in vivo.

Tissue	1 (LA)	2 (LA + 702258)	3 (702258)	5 (Placebo)
Liver	0.01 ± 0.01 ²	0.04 ± 0.03 ^{1,3,5}	0.01 ± 0.01 ²	ND ²
White Adipose	ND	0.01 ± 0.02	ND	ND
Colon	ND	0.03 ± 0.04	ND	ND
Cecum digesta	0.08 ± 0.02	0.16 ± 0.23	0.06 ± 0.04	0.07 ± 0.06
Faeces	0.09	0.15	0.04	0.09

- 5 Data are expressed as mean ± SD (n = 8), g / 100g FAME. ^{1,2,3,5} Different superscript numbers within a column indicate significant difference (n=8, P< 0.05). ND = not detected.

Table IV. Fatty acid composition (%) of liver and adipose tissue from BALB/c mice

FAME	Liver	Adipose tissue							
		A	B	C	D	A	B	C	D
C16:0	23.11 ± 0.99 ^{c,d}	23.89 ± 0.71 ^{c,d}	27.78 ± 0.64 ^{a,b}	27.64 ± 0.42 ^{a,b}	28.84 ± 2.83 ^c	28.73 ± 2.16 ^c	30.92 ± 0.82 ^{a,b,d}	29.87 ± 0.83 ^c	
C16:1c9	1.56 ± 0.10 ^{b,c,d}	2.07 ± 0.18 ^{a,d}	2.32 ± 0.18 ^a	2.73 ± 0.27 ^{a,b}	5.39 ± 0.73	6.07 ± 2.01	6.69 ± 1.95	6.54 ± 2.31	35
C18:0	13.83 ± 0.42 ^{b,c,d}	12.36 ± 0.44 ^{a,d}	11.50 ± 0.35 ^{a,d}	10.40 ± 0.35 ^{a,b,c}	8.76 ± 1.00	8.00 ± 2.33	7.71 ± 1.99	7.31 ± 2.87	
C18:1c9	9.16 ± 0.36 ^{c,d}	10.16 ± 0.62 ^{c,d}	13.75 ± 0.57 ^{a,b}	15.16 ± 0.75 ^{a,b}	14.41 ± 1.29	14.83 ± 3.01	16.92 ± 3.07	18.35 ± 4.84	
C18:2n-6	18.70 ± 0.35	18.32 ± 0.21 ^d	18.21 ± 0.32 ^d	19.38 ± 0.42 ^{b,c}	18.22 ± 0.25 ^c	18.80 ± 0.81 ^c	16.56 ± 0.77 ^{a,b}	17.96 ± 3.39	
C18:3n-3	8.37 ± 0.91 ^{c,d}	9.47 ± 0.64 ^{c,d}	0.50 ± 0.03 ^{a,b}	0.58 ± 0.04 ^{a,b}	6.56 ± 1.00 ^{c,d}	7.35 ± 2.15 ^{c,d}	0.66 ± 0.10 ^{a,b}	0.79 ± 0.28 ^{a,b}	
C18:3n-6	0.20 ± 0.02 ^{c,d}	0.17 ± 0.01 ^{c,d}	0.30 ± 0.03 ^{a,b}	0.32 ± 0.01 ^{a,b}	0.15 ± 0.02	0.13 ± 0.03	0.15 ± 0.03	0.14 ± 0.04	
C18:4n-3	0.14 ± 0.01 ^{c,d}	0.16 ± 0.01 ^{c,d}	0.23 ± 0.03 ^{a,b}	0.22 ± 0.02 ^{a,b}	0.16 ± 0.02	0.12 ± 0.03	0.14 ± 0.04	0.14 ± 0.03	
C20:3n-6	0.73 ± 0.05 ^b	0.61 ± 0.02 ^a	0.69 ± 0.04	0.63 ± 0.03	0.37 ± 0.04	0.30 ± 0.09	0.32 ± 0.09	0.29 ± 0.16	
C20:4n-6	6.71 ± 0.29 ^{b,c,d}	5.57 ± 0.14 ^{a,c,d}	11.45 ± 0.58 ^{a,c,d}	9.78 ± 0.53 ^{a,b,c}	6.20 ± 0.97 ^c	5.27 ± 2.04 ^{c,d}	10.57 ± 3.45 ^{a,b}	9.29 ± 5.27 ^b	

C20:5n-3	3.77 ± 0.30 ^{b,c,d}	3.07 ± 0.12 ^{a,c,d}	0.19 ± 0.01 ^{a,b}	0.17 ± 0.02 ^{a,b}	2.86 ± 0.27 ^{c,d}	2.37 ± 0.76 ^{c,d}	0.29 ± 0.07 ^{a,b}	0.23 ± 0.15 ^{a,b}
C22:5n-3	1.02 ± 0.06 ^{c,d}	0.94 ± 0.05 ^{c,d}	0.23 ± 0.02 ^{a,b}	0.26 ± 0.02 ^{a,b}	0.67 ± 0.06 ^{c,d}	0.55 ± 0.14 ^{c,d}	0.27 ± 0.03 ^{a,b}	0.27 ± 0.16 ^{a,b}
C22:6n-3	6.56 ± 0.35 ^d	5.66 ± 0.28 ^d	5.51 ± 0.41	4.76 ± 0.24 ^{a,b}	1.49 ± 0.12	1.17 ± 0.36	1.22 ± 0.30	1.10 ± 0.59
n-6/n-3	1.36 ± 0.10 ^{c,d}	1.30 ± 0.07 ^{c,d}	4.70 ± 0.23 ^{a,b}	5.07 ± 0.16 ^{a,b}	2.20 ± 0.21 ^{c,d}	2.15 ± 0.15 ^{c,d}	10.80 ± 0.28 ^{a,b}	11.56 ± 0.92 ^{a,b}

Results are expressed as means (SEM) g/100g FAME (n=8). ^{a, b, c, d} Different superscript numbers within a column indicate significant difference

(n = 8, p<0.05). FAME = fatty acid methyl esters. Group A = 1% α -linolenic acid in combination with 1×10⁹ live *B. breve* NCIMB 702258 per

day. group B = 1% α -linolenic acid, Group C = standard diet in combination with 1×10⁹ live *B. breve* NCIMB 702258, and group D =

unsupplemented mice. C16:0 palmitic acid; C16:1c9 palmitoleic acid; C18:0 stearic acid; C18:1c9 oleic acid; C18:2n-6 linoleic acid; C18:3n-3

linolenic acid; C18:3n-6 γ -linolenic acid; C18:4n-3 stearidonic acid; C20:3n-6 dihomo- γ -linolenic acid; C20:4n-6 arachidonic acid; C20:5n-3

eicosapentaenoic acid; C22:5n-3 docosapentaenoic acid; C22:6n-3 docosahexaenoic acid.

Table V. Fatty acid composition (%) of brain from BALB/c mice

FAME	Brain			
	A	B	C	D
C16:0	30.69 ± 0.65	32.38 ± 0.44 ^c	28.88 ± 0.90 ^{b,d}	31.47 ± 0.51 ^c
C16:1c9	0.81 ± 0.02 ^{a,b}	0.94 ± 0.03 ^{a,c,d}	0.73 ± 0.03 ^{a,d}	0.84 ± 0.03 ^{b,c}
C18:0	20.22 ± 0.07 ^b	19.87 ± 0.14 ^a	20.11 ± 0.20	19.85 ± 0.28
C18:1c9	17.58 ± 0.19	17.61 ± 0.24	17.14 ± 0.29	17.23 ± 0.33
C18:2n-6	1.18 ± 0.04 ^{b,c}	1.54 ± 0.10 ^{a,c,d}	1.00 ± 0.05 ^{a,b}	1.14 ± 0.03 ^b
C18:3n-3	0.10 ± 0.01 ^{b,c,d}	0.18 ± 0.02 ^{a,c,d}	ND	ND
C18:3n-6	ND	ND	ND	ND
C18:4n-3	ND	ND	ND	ND
C20:3n-6	ND	ND	ND	ND
C20:4n-6	7.06 ± 0.07 ^c	6.88 ± 0.12 ^{c,d}	7.84 ± 0.23 ^{a,b}	7.37 ± 0.17 ^b
C20:5n-3	0.17 ± 0.02 ^{c,d}	0.15 ± 0.01 ^{c,d}	0.04 ± 0.01 ^{a,b}	0.07 ± 0.01 ^{a,b}
C22:5n-3	0.25 ± 0.01 ^{c,d}	0.24 ± 0.01 ^c	0.11 ± 0.01 ^{a,b}	0.17 ± 0.04 ^a
C22:6n-3	9.95 ± 0.30 ^{b,d}	8.87 ± 0.30 ^a	10.15 ± 0.75	8.74 ± 0.43 ^a
n-6/n-3	0.79 ± 0.03 ^{b,d}	0.89 ± 0.03 ^a	0.87 ± 0.04	0.97 ± 0.04 ^a

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Results are expressed as means (SEM) g/100g FAME (n=8). ^{a,b,c,d} Different superscript numbers within a column indicate significant difference (n=8, p<0.05). Group A = 1% α-linolenic acid in combination with 1×10⁹ live *B. breve* NCIMB 702258 per day. group B = 1% α-linolenic acid, Group C = standard diet in combination with 1×10⁹ live *B. breve* NCIMB 702258, and group D = unsupplemented diet. ND = not detected.

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Claims:

1. Use of a CLA –producing bacterium for the *in vivo* conversion in the gut of polyunsaturated fatty acids to CLA.
- 5 2. Use as claimed in claim 1 wherein the CLA-producing bacterium is selected from one or more of the group consisting of propionibacteria, lactobacilli, lactococci and streptococci , and bifidobacteria.
3. Use as claimed in claim 1 or 2 wherein the CLA-producing bacterium is one or more of *Bifidobacterium breve* as deposited at the National Culture of
10 Industrial and Marine Bacteria under the accession nos. 702258, *B. breve* DPC 6330 as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41497 on 28th September 2007, or *B. longum* DPC 6315 as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41508 on 18th October 2007.
- 15 4. Use of a CLA –producing bacterium to alter the fatty acid composition of internal organs of the body.
5. Use as claimed in claim 4 wherein the CLA-producing bacterium is selected from one or more of the group consisting of propionibacteria, lactobacilli, lactococci and streptococci, and bifidobacteria.
- 20 6. Use as claimed in claim 4 or 5 wherein the CLA-producing bacterium is one or more of *Bifidobacterium breve* as deposited at the National Culture of Industrial and Marine Bacteria under the accession nos. 702258, *B. breve* DPC 6330 as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41497 on 28th September 2007, or *B. longum* DPC 6315 as
25 deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41508 on 18th October 2007.
7. Use as claimed in any of claims 4 to 6 wherein the organ is the liver.
8. Use of a CLA –producing bacterium in the preparation of a composition for the treatment of inflammatory diseases.
- 30 9. Use as claimed in claim 8 wherein the CLA-producing bacterium is selected from one or more of the group consisting of propionibacteria , lactobacilli, lactococci and streptococci , and bifidobacteria.

10. Use as claimed in claim 8 or 9 wherein the CLA-producing bacterium is one or more of *Bifidobacterium breve* as deposited at the National Culture of Industrial and Marine Bacteria under the accession nos. 702258, *B. breve* DPC 6330 as deposited at the National Culture of Industrial and Marine Bacteria under
5 the accession no. 41497 on 28th September 2007, or *B. longum* DPC 6315 as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41508 on 18th October 2007.

11. A composition comprising a CLA producing organism together with pharmaceutically acceptable or nutritionally acceptable additives.

10 12. A composition as claimed in claim 11 wherein the CLA-producing bacterium is selected from one or more of the group consisting of propionibacteria, lactobacilli, lactococci and streptococci, and bifidobacteria.

13. A composition as claimed in claim 11 or 12 wherein the CLA-producing bacterium is one or more of *Bifidobacterium breve* as deposited at the National
15 Culture of Industrial and Marine Bacteria under the accession nos. 702258, *B. breve* DPC 6330 as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41497 on 28th September 2007, or *B. longum* DPC 6315 as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41508 on 18th October 2007.

20 14. A probiotic composition as claimed in any of claims 11 to 13 which is a foodstuff selected from a yogurt, a yogurt drink, a cheese, a milk, a spread, a fruit juice, a water which is either flavoured or unflavoured.

15. A probiotic composition as claimed in any of claims 11 to 14 further comprising a substrate, which can be converted into a bioactive compound *in vivo* by the CLA producing organism.
25

16. A probiotic composition as claimed in claim 15 when the substrate is a polyunsaturated fatty acid.

17. A probiotic composition as claimed in claim 16 wherein the polyunsaturated fatty acid is linoleic acid, linolenic acid, oleic acid, palmitic acid
30 or stearic acid.

18. A probiotic composition as claimed in any of claims 11 to 17 which can reduce gut inflammation in diseases such as Inflammatory Bowel Syndrome or Inflammatory Bowel Disease, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease, eczema, asthma or psychiatric diseases such as depression.
- 5 19. A method of converting dietary polyunsaturated fatty acids to CLA *in vivo* comprising administration to a subject of a live CLA-producing bacterial strain.
20. A method of altering the fatty acid composition of internal organs of the body comprising administering to a subject a live CLA-producing bacterial
- 10 strain.
21. A method to drive DHA (docosahexaenoic acid) /EPA (eicosapentaenoic acid) incorporation into host tissues using dietary CLA or a strain producing CLA, in order to improve memory loss and cognition.
22. B. breve DPC 6330 as deposited at the National Culture of Industrial and
- 15 Marine Bacteria under the accession no. 41497 on 28th September 2007.
23. B. longum DPC 6315 as deposited at the National Culture of Industrial and Marine Bacteria under the accession no. 41508 on 18th October 2007.
24. A feedstuff for animals comprising a CLA producing organism together with nutritionally acceptable ingredients.
- 20 25. A feedstuff as claimed in claim 24 further comprising a substrate which can be converted into a bioactive compound *in vivo* by the CLA producing organism.

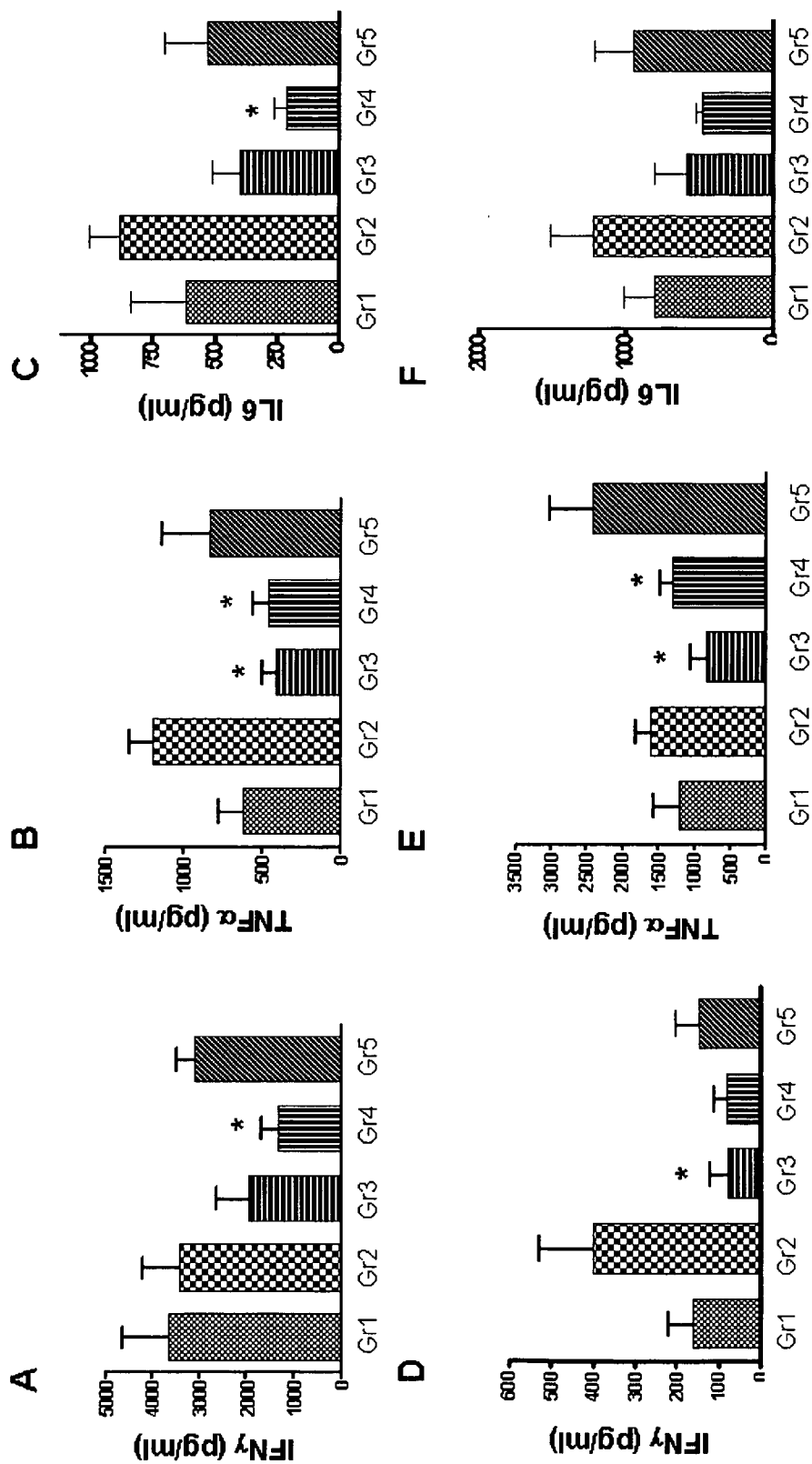


Figure 1

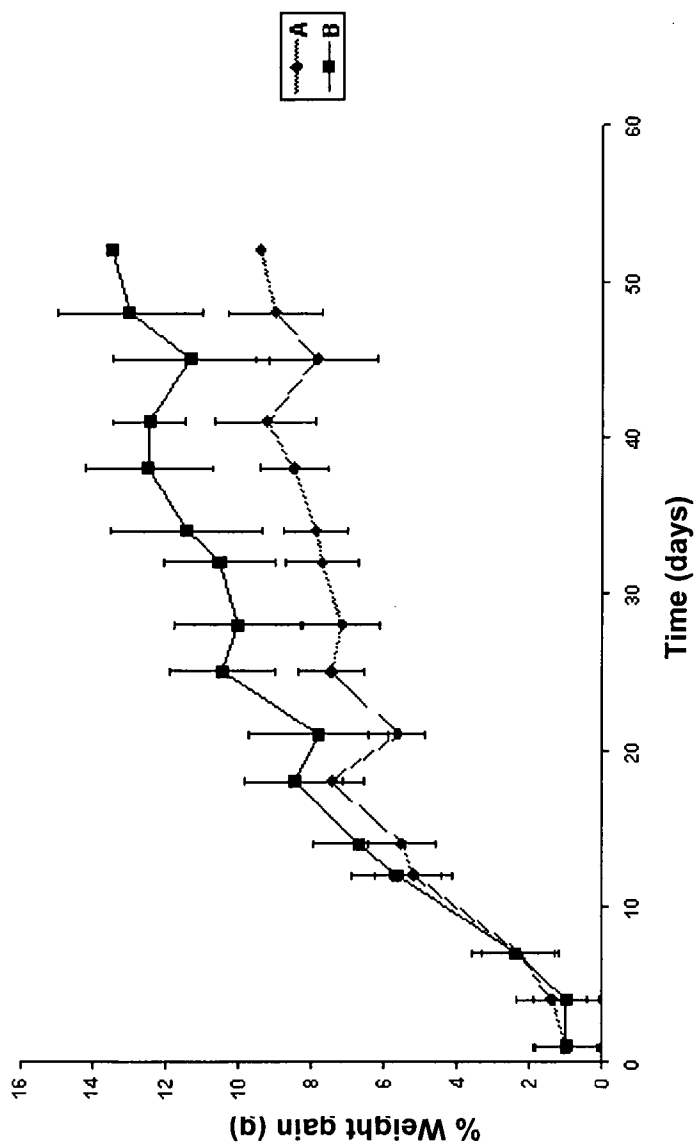


Figure 2

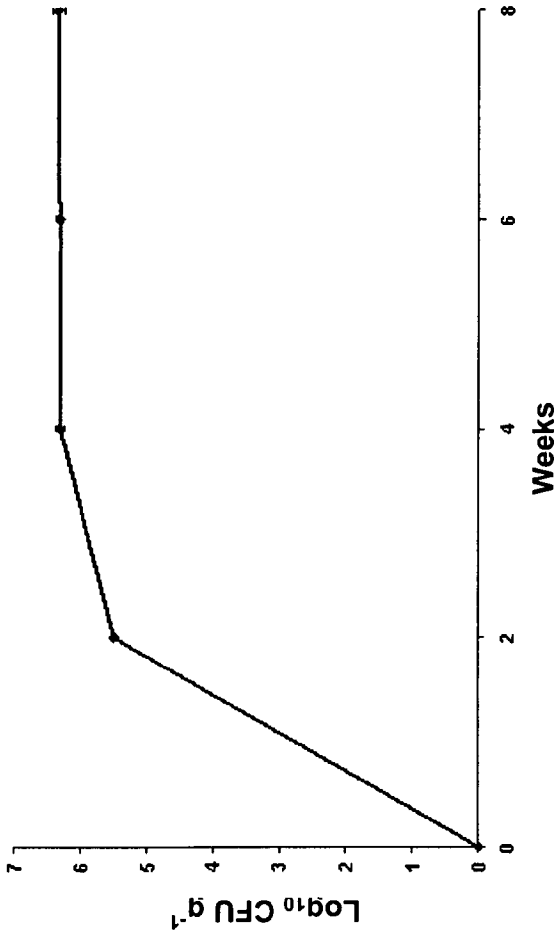


Figure 3

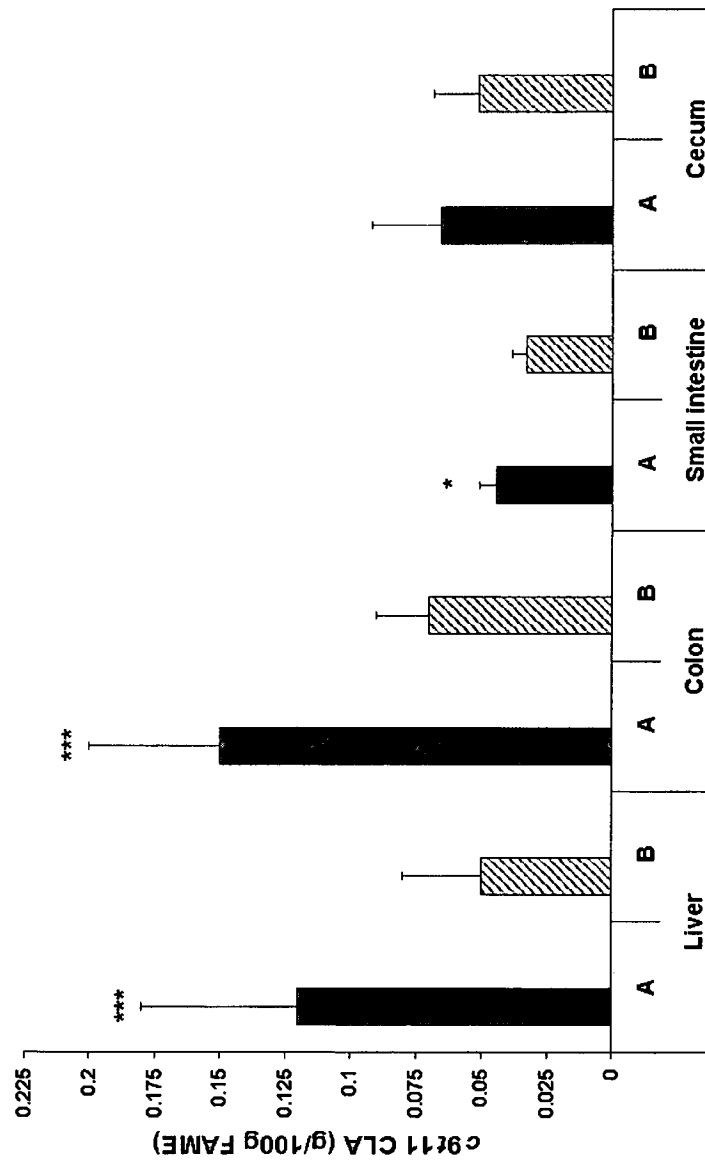


Figure 4

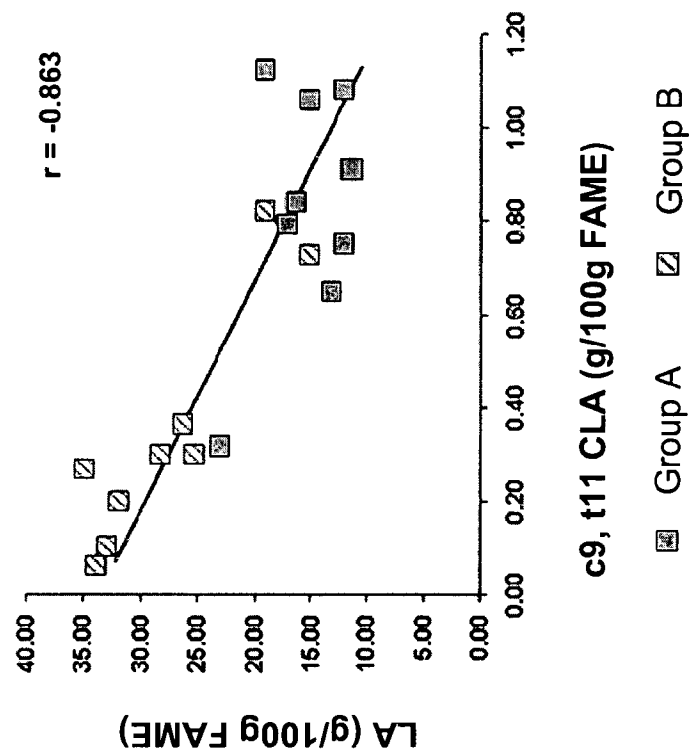


Figure 5

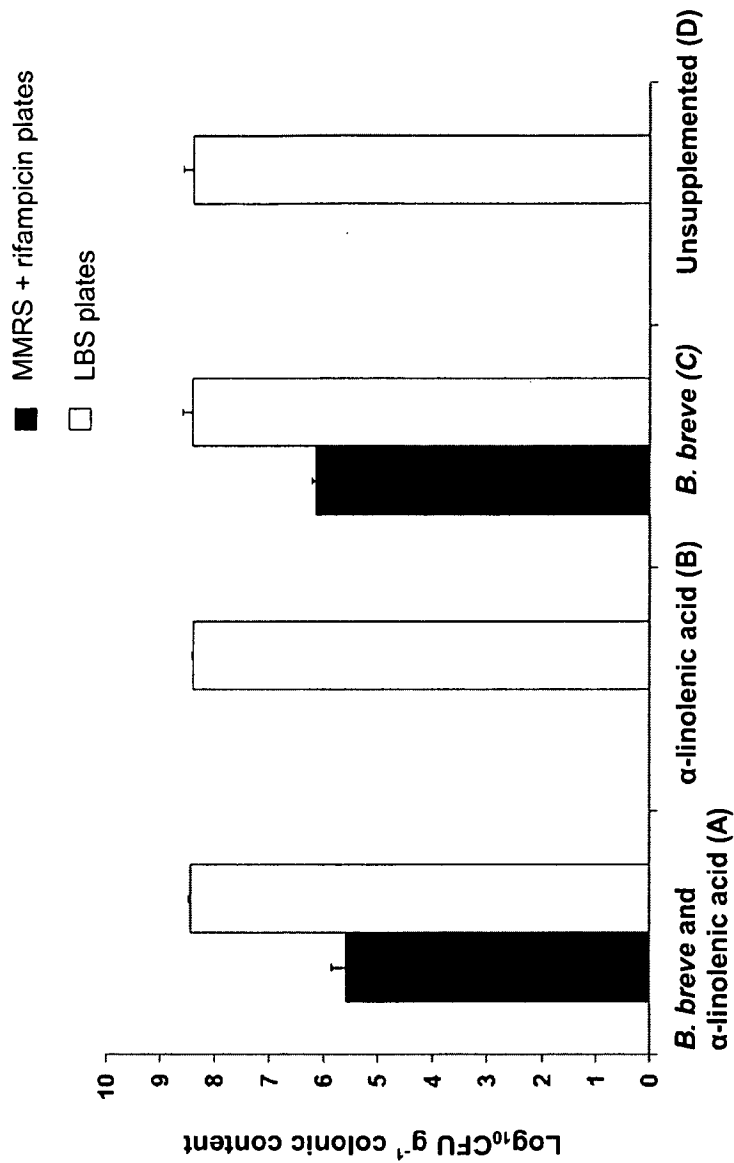


Figure 6

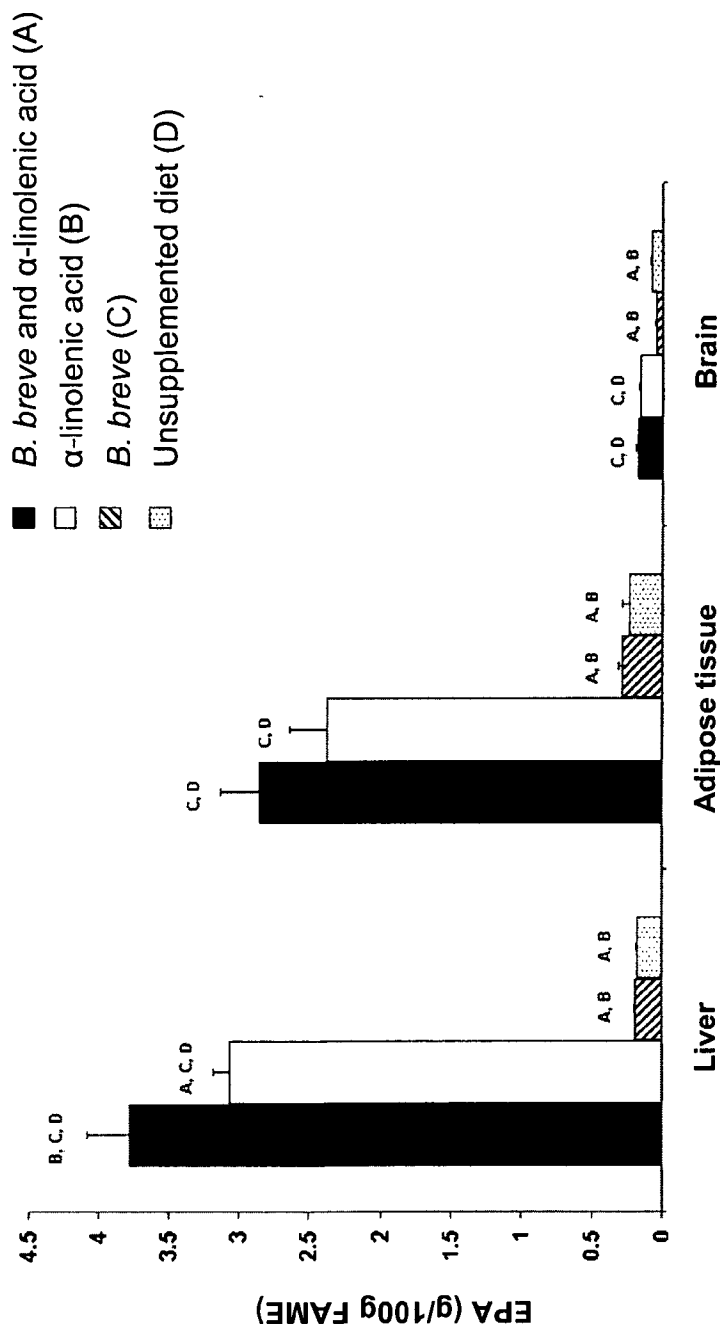


Figure 7

- *B. breve* and α -linolenic acid (A)
- α -linolenic acid (B)
- ▨ *B. breve* (C)
- ▤ Unsupplemented diet (D)

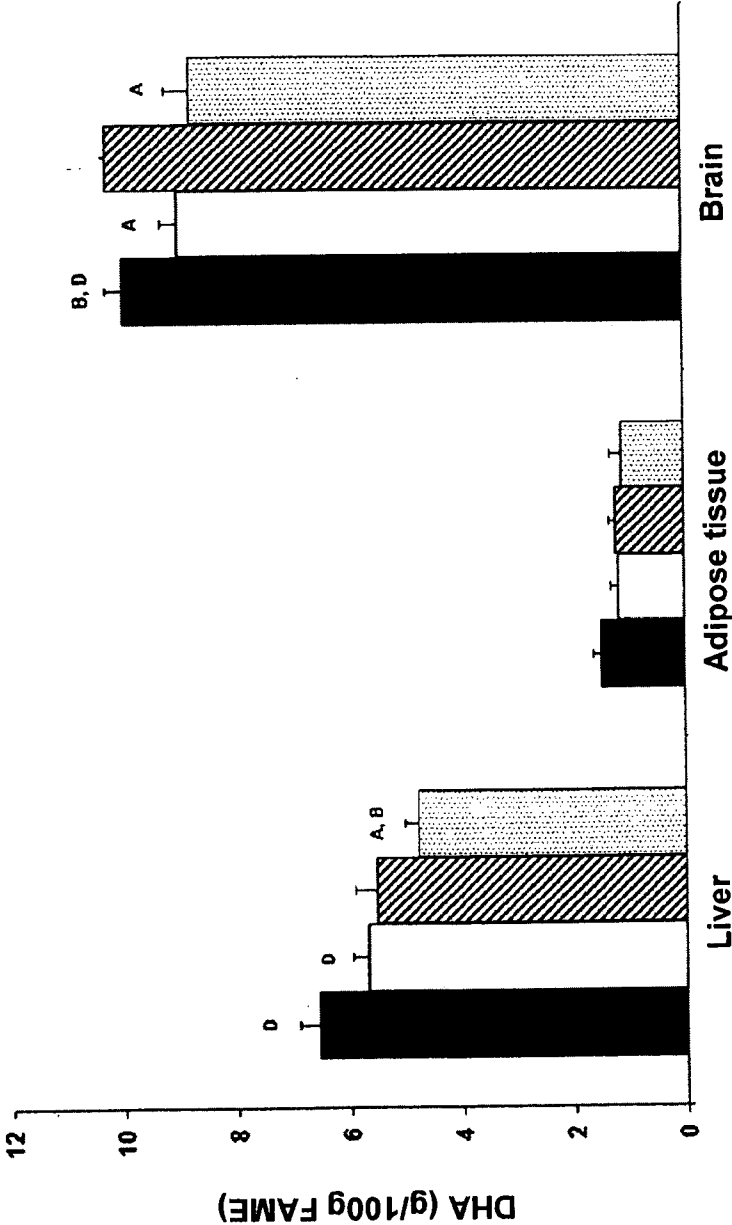


Figure 8