

GRAS Notice (GRN) No. 453

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

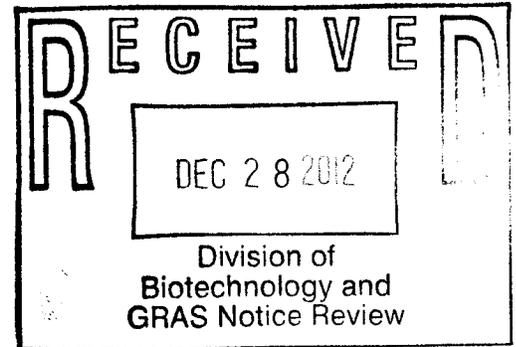


ORIGINAL SUBMISSION

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December 21, 2012

Paulette Gaynor, Ph.D.
Office of Food Additive Safety
HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740



Dear Dr. Gaynor,

Enclosed are three copies of the updated GRAS Exemption Claim and page 24, Table 8 The Proposed Conventional Food Categories for the Addition of *Bifidobacterium breve* M-16V, for the previously submitted "Generally Recognized As Safe (GRAS) Determination For *Bifidobacterium breve* M-16V in Selected Conventional and Medical Foods" that was prepared by Spherix Consulting, Inc. for Morinaga Milk Industry Co., Ltd. We have inserted "or sent to FDA upon request" at the end of section I.E of the GRAS Exemption Claim and removed "meat sticks" from the proposed food categories. Importantly, removing the meat sticks did not affect the Estimated Daily Intakes or require any changes be made to the remaining portion of the Intended Uses and Estimated Intakes of *B. breve* M-16V section.

Please contact me at 301-897-0611, 240-565-5501, or clairek@chromadex.com if you have any questions or concerns.

Sincerely,

(b) (6)

Claire L. Kruger, Ph.D., D.A.B.T.
President

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I. GRAS EXEMPTION CLAIM

A. NAME AND ADDRESS OF SPONSOR

1. Business Address

Morinaga Milk Industry Co., Ltd.
33-1, Shiba 5-Chome, Minato-ku
Tokyo 108-8384, Japan
Tel: 81-3-3798-0152
Fax: 81-3-3798-0107

2. Email address

interntl@morinagamilk.co.jp

B. COMMON OR USUAL NAME OF GRAS SUBSTANCE

The subject of this Generally Recognized As Safe (GRAS) determination is *Bifidobacterium breve* (*B. breve*) M-16V, which is owned and distributed by Morinaga Milk Industry Co., Ltd.

C. INTENDED USE

Morinaga Milk Industry Co., Ltd. intends to add *B. breve* M-16V to selected food products for the general population and medical foods. The amount of *B. breve* M-16V will not exceed 5×10^9 colony forming units (cfu) per serving of selected foods and 10^8 cfu/g of medical food.

D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of *B. breve* M-16V for the intended uses specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). To demonstrate that *B. breve* M-16V is safe, and GRAS, under the intended conditions of use, the safety of the intake of *B. breve* M-16V has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

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The proposed use of *B. breve* M-16V as an ingredient in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. Bifidobacteria are naturally occurring gut microbiota in infants and adults. *B. breve* has been detected in the stools of infants and adults.
2. Bifidobacteria have been consumed in fermented foods for decades and current commercial strains include *Bifidobacterium animalis* (*B. animalis*) ssp. *lactis* strain Bf-6, *Bifidobacterium lactis* (*B. lactis*) Bb-12, *B. lactis* DR10 (HN019), *Bifidobacterium longum* (*B. longum*) BB536, *B. breve* Yakult, *B. breve* SBT-2928, and *B. breve* C50. *B. breve* M-16V was first commercially available in Japan in 1976.
3. In the United States, various *Bifidobacterium* species have been determined to be GRAS for use in conventional foods and infant formulas, including: *B. animalis* ssp. *lactis* Bf-6 for use in selected foods (GRN 377; 10^{11} cfu/serving of conventional foods); *B. lactis* Bb-12 for use in infant formulas for four months-of-age and older (GRN 49; 10^7 - 10^8 cfu/g infant formula) and *B. longum* BB536 for use in selected foods and infant formulas (GRN 268; 10^{10} cfu/serving of conventional foods; 10^{10} cfu/g of term infant formula).
4. *Bifidobacterium breve* M-16V is a Gram-positive anaerobic bacterium. This organism was deposited with the Belgian Co-ordinated Collections of Microorganisms (BCCM) and designated LMG 23729.
5. The original frozen culture of *B. breve* M-16V is tightly controlled to ensure purity and genetic stability of the strain.
6. Product specifications assure that *B. breve* M-16V is suitable for use in selected conventional and medical foods.
7. Finished products made with *B. breve* M-16V cultures reproducibly meet compositional standards and comply with limits on contaminants appropriate for food-grade ingredients.
8. *B. breve* M-16V meets the safety standards enumerated by the Food and Agriculture Organization of the United Nations/World Health Organization's (FAO/WHO) guidelines for the evaluation of microbes for probiotic use in foods. Results show that *B. breve* M-16V is not toxic or pathogenic and is therefore safe for use in foods:

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- a. The genome of *B. breve* M-16V does not contain regions with significant homology to known antibiotic resistance genes, including those found in other strains of bifidobacteria and lactobacilli.
 - b. Functional assays indicate that *B. breve* M-16V exhibits antibiotic susceptibility patterns similar to the type strain *B. breve* ATCC 15700.
 - c. *B. breve* M-16V does not contain plasmids.
 - d. *B. breve* M-16V produces predominantly L-lactic acid, while production of D-lactic acid is negligible.
 - e. *B. breve* M-16V hydrolyzes the conjugated bile acids taurocholic and glycocholic acid to the primary bile acid cholic acid and hydrolyzed glycochenodeoxycholic and taurochenodeoxycholic acid to chenodeoxycholic acid.
 - f. *B. breve* M-16V does not dehydroxylate cholic acid and chenodeoxycholic acid to the secondary bile acids deoxycholic and lithocholic acid.
 - g. *B. breve* M-16V does not produce biogenic amines.
 - h. *B. breve* M-16V does not produce ammonia.
 - i. *B. breve* M-16V does not have azoreductase or nitroreductase activity.
 - j. *B. breve* M-16V does not have hemolytic activity.
 - k. *B. breve* M-16V has no deleterious effects on platelet aggregation or viability.
 - l. *B. breve* M-16V has no mucolytic activity.
9. Toxicology studies show no evidence of *B. breve* M-16V translocating the gut epithelium, it does not induce mutations in *Salmonella typhimurium* strain TA98 and TA100 with or without metabolic activation, and does not produce test article-related toxicity in a 90-day repeated dose oral toxicity study in rats administered 2.3×10^{11} cfu *B. breve* M-16V/kg/day.
10. Twelve studies published from 1992 – 2012 have reported the safe administration of *B. breve* M-16V at doses up to 1.5×10^{10} cfu/d for durations of up to 3 months in a total of 430 health compromised and/or premature infants. *B. breve* M-16V was well tolerated and no treatment-related adverse health effects were noted.

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11. *B. breve* M-16V strain has been administered in two studies in adults at a dose of 2×10^{10} cfu/d for 4-8 wk in patients with intermittent to mild persistent asthma or atopic dermatitis. There were no treatment-related adverse effects noted.
12. Other non-M-16V strains of *B. breve* have been administered to adults and children at doses up to 8×10^{11} cfu/d for durations up to 1 yr and no adverse treatment-related effects were reported.
13. Assuming a maximum addition of 5.0×10^9 cfu *B. breve* M-16V/serving of the conventional foods listed in this document, the estimated mean and 90th percentile 2-day average intakes of *B. breve* M-16V from all categories combined in the population ages 2 years and older are 3.8×10^{10} and 6.0×10^{10} cfu/day, respectively.
14. Under the most conservative assumptions, a maximum addition of 10^8 cfu of *B. breve* M-16V per gram of powdered medical foods containing hydrolyzed proteins and/or amino acid mixtures for children ages one to ten years old, the estimated daily intake of a one year-old and a ten year-old is 1.6×10^{10} cfu and 4.2×10^{10} cfu *B. breve* M-16V per day, respectively.

Determination of the GRAS status of *B. breve* M-16V under the intended conditions of use has been made through the deliberations of A. Wallace Hayes, PhD, DABT, FATS, ERT, CNS, FACN; Gregor Reid, PhD, MBA, ARM, CCM, Dr HS, FCAHS; Roger Clemens, Dr PH, CNS, FACN, FIFT. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of *B. breve* M-16V and the human exposure to *B. breve* M-16V resulting from its intended use as an ingredient in selected conventional foods and medical foods containing hydrolyzed proteins and/or amino acid mixtures for children, and have concluded:

There is no evidence in the available information on B. breve M-16V that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when B. breve M-16V is used at levels that might reasonably be expected from the proposed applications. B. breve M-16V is GRAS for use in selected conventional and medical foods as proposed by Morinaga Milk Industry Co., Ltd.

Therefore, *B. breve* M-16V is safe and GRAS at the proposed levels of addition to foods. *B. breve* M-16V is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

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E. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., President, Spherix Consulting, Incorporated, 6430 Rockledge Drive, Westmoreland Bldg, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ClaireK@chromadex.com or be sent to FDA upon request.

F. SIGNATURE

Pursuant to the criteria provided in proposed 21 CFR 170.36, Morinaga Milk Industry Co., Ltd. hereby notifies the Food and Drug Administration that the use of *B. breve* M-16V in foods under the intended conditions of use is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Morinaga Milk Industry Co., Ltd. has determined that such use is Generally Recognized As Safe through scientific procedures.

(b) (6)

Signature
Claire L. Kruger, Authorized Representative of
Morinaga Milk Industry Co., Ltd.

December 14, 2012

Date

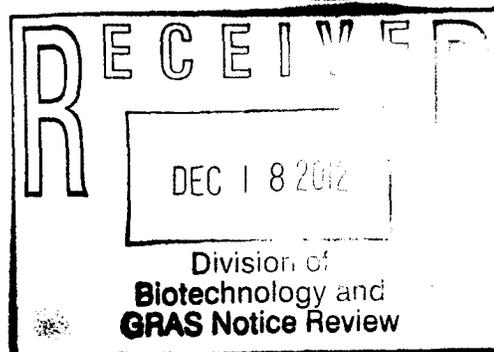
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Table 8. Proposed Conventional Food Categories for the Addition of <i>Bifidobacterium breve</i> M-16V	
Dairy products/dairy-based foods and dairy substitutes	<ol style="list-style-type: none"> 1. Skim milk 2. Cheese spreads 3. Cheese, imitation 4. Cheese, processed 5. Cream substitutes 6. Cream, heavy 7. Fermented milk (flavored, heat treated), including buttermilk, kefir, and flavored milk beverage mixes 8. Frozen desserts, including ice cream, ice milk, frozen yogurt, frozen novelties, and imitation milk 9. Meal replacements, liquids and dry mixes 10. Milk shakes 11. Milk (plain and flavored), including cocoa, chocolate milk, fruit milks, coffee drinks (fluid/dry) 12. Puddings and custards 13. Smoothies 14. Whipped toppings 15. Yogurt 16. Butter and dried milk products 17. Milk powder for pregnant women, plain and flavored 18. Milk powder for adult people, plain and flavored 19. Milk powder for elderly people, plain and flavored
Miscellaneous	<ol style="list-style-type: none"> 1. Candies, including hard candies, mints, chocolate and all other types of confections (i.e., chewing gum), cocoa powder, condiment sauces, (i.e., catsup, BBQ, taco, steak, cocktail, Worcestershire, teriyaki, cheese-based, hollandaise, tartar, béarnaise) 2. Gelatin desserts, plain or with fruit gravies 3. Peanut and other nut butters/spreads 4. Snack foods, including chips, popcorn mixtures 5. Weaning foods, including meals, desserts, fruits, cereal, vegetables, snacks, juices

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December 14, 2012

Office of Food Additive Safety
HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740



To Whom It May Concern:

Enclosed please find three copies of "Generally Recognized As Safe (GRAS) Determination For *Bifidobacterium breve* M-16V in Selected Conventional and Medical Foods" and the accompanying appendices. This GRAS notification has been prepared by Spherix Consulting, Inc. for Morinaga Milk Industry Co., Ltd.

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix Consulting, Inc., 6430 Rockledge Drive, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Mobile: 240-565-5501; Facsimile: 301-897-2567; Email: ClaireK@chromadex.com.

Should you have any questions or concerns, please contact me at the number listed above.

Sincerely,

(b) (6)

Claire L. Kruger, Ph.D., D.A.B.T.
President

Enclosures:

Three copies of the dossier entitled "Generally Recognized As Safe (GRAS) Determination For *Bifidobacterium breve* M-16V in Selected Conventional and Medical Foods" and the accompanying appendices

Three copies of the GRAS Panel Consensus Statement for the above-referenced GRAS Notification

**Generally Recognized As Safe (GRAS) Determination For
Bifidobacterium breve M-16V in
Selected Conventional and Medical Foods**

Prepared for:

Morinaga Milk Industry Co., Ltd.
33-1, Shiba 5-Chome
Minato-ku, Tokyo 108-8384
Japan

Prepared by:

Spherix Consulting, Inc.
6430 Rockledge Drive #503
Bethesda, MD 20817
USA

September 18, 2012

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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Definition</u>
ABCD-2	thymus and activation regulated cytokine (aka CCL-17 and TARC)
A/G	albumin/globulin ratio
ALP	alkaline phosphatase
ALT	alanine aminotransferase
APTT	activated partial thromboplastin time
AST	aspartate aminotransferase
ATCC	American Type Culture Collection
BCCM	Belgian Co-ordinated Collections of Micro-Organizations
BLASTN	Basic Local Alignment Search Tool for Nucleotides
BUN	blood urea nitrogen
Ca	calcium
CCL-17	thymus and activation regulated cytokine (aka ABCD-2 and TARC)
CFP	carbohydrate fermentation pattern
CFU	colony-forming units
Cl	chloride
CRP	C-reactive protein
CSF	cerebrospinal fluid
CTACK	cutaneous T cell-attracting chemokine
FAO	Food and Agriculture Organization of the United Nations
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FFDCA	Federal Food, Drug, and Cosmetic Act
FOS	fructo-oligosaccharide
GOS	galacto-oligosaccharide
GRAS	Generally Recognized as Safe
HACCP	Hazard Analysis and Critical Control Point
Hb	hemoglobin
Hct	hematocrit
IBD	inflammatory bowel disease

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IBS	irritable bowel syndrome
IGS	International Genetic Standardization
K	potassium
lc-FOS	long-chain fructo-oligosaccharides
LDH	lactate dehydrogenase
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MIC	minimum inhibitory concentration
MPO	myeloperoxidase
Na	sodium
NCBI	National Center for Biotechnology Information
NCHS	National Center for Health Statistics
NEC	necrotizing enterocolitis
NHANES	National Health and Nutrition Examination Survey
NICU	Neonatal Intensive Care Unit
NK	natural killer cell counts
NTED	Neonatal toxic shock syndrome (TSS) - exanthematous disease
ORFs	open-reading frames
P	inorganic phosphorus
PABA	para-aminoenzoic acid
PCR	polymerase chain reaction
PL	phospholipids
PTT	prothrombin time
RAPD-PCR	random amplification of polymorphic DNA polymerase chain reaction
RBC	red blood cell count
sc-GOS	short-chain galacto-oligosaccharides
SCORAD	SCORing Atopic Dermatitis
SIRS	systemic inflammatory response syndrome
TARC	thymus and activation regulated cytokine (aka ABCD-2 and CCL-17)

T-bil	total bilirubin
TC	total cholesterol
TG	triglycerides
TP	total protein
TSS	toxic shock syndrome
USDA	U.S. Department of Agriculture
WBC	white blood cell count
WHO	World Health Organization
γ -GTP	γ -glutamyl transpeptidase

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I. GRAS EXEMPTION CLAIM

A. NAME AND ADDRESS OF SPONSOR

1. Business Address

Morinaga Milk Industry Co., Ltd.
33-1, Shiba 5-Chome, Minato-ku
Tokyo 108-8384, Japan
Tel: 81-3-3798-0152
Fax: 81-3-3798-0107

2. Email address

interntl@morinagamilk.co.jp

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The proposed use of *B. breve* M-16V as an ingredient in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. Bifidobacteria are naturally occurring gut microbiota in infants and adults. *B. breve* has been detected in the stools of infants and adults.
2. Bifidobacteria have been consumed in fermented foods for decades and current commercial strains include *Bifidobacterium animalis* (*B. animalis*) ssp. *lactis* strain Bf-6, *Bifidobacterium lactis* (*B. lactis*) Bb-12, *B. lactis* DR10 (HN019), *Bifidobacterium longum* (*B. longum*) BB536, *B. breve* Yakult, *B. breve* SBT-2928, and *B. breve* C50. *B. breve* M-16V was first commercially available in Japan in 1976.
3. In the United States, various *Bifidobacterium* species have been determined to be GRAS for use in conventional foods and infant formulas, including: *B. animalis* ssp. *lactis* Bf-6 for use in selected foods (GRN 377; 10^{11} cfu/serving of conventional foods); *B. lactis* Bb-12 for use in infant formulas for four months-of-age and older (GRN 49; 10^7 - 10^8 cfu/g infant formula) and *B. longum* BB536 for use in selected foods and infant formulas (GRN 268; 10^{10} cfu/serving of conventional foods; 10^{10} cfu/g of term infant formula).
4. *Bifidobacterium breve* M-16V is a Gram-positive anaerobic bacterium. This organism was deposited with the Belgian Co-ordinated Collections of Micro-organisms (BCCM) and designated LMG 23729.
5. The original frozen culture of *B. breve* M-16V is tightly controlled to ensure purity and genetic stability of the strain.
6. Product specifications assure that *B. breve* M-16V is suitable for use in selected conventional and medical foods.
7. Finished products made with *B. breve* M-16V cultures reproducibly meet compositional standards and comply with limits on contaminants appropriate for food-grade ingredients.
8. *B. breve* M-16V meets the safety standards enumerated by the Food and Agriculture Organization of the United Nations/World Health Organization's (FAO/WHO) guidelines for the evaluation of microbes for probiotic use in foods. Results show that *B. breve* M-16V is not toxic or pathogenic and is therefore safe for use in foods:

000018

- a. The genome of *B. breve* M-16V does not contain regions with significant homology to known antibiotic resistance genes, including those found in other strains of bifidobacteria and lactobacilli.
 - b. Functional assays indicate that *B. breve* M-16V exhibits antibiotic susceptibility patterns similar to the type strain *B. breve* ATCC 15700.
 - c. *B. breve* M-16V does not contain plasmids.
 - d. *B. breve* M-16V produces predominantly L-lactic acid, while production of D-lactic acid is negligible.
 - e. *B. breve* M-16V hydrolyzes the conjugated bile acids taurocholic and glycocholic acid to the primary bile acid cholic acid and hydrolyzed glycochenodeoxycholic and taurochenodeoxycholic acid to chenodeoxycholic acid.
 - f. *B. breve* M-16V does not dehydroxylate cholic acid and chenodeoxycholic acid to the secondary bile acids deoxycholic and lithocholic acid.
 - g. *B. breve* M-16V does not produce biogenic amines.
 - h. *B. breve* M-16V does not produce ammonia.
 - i. *B. breve* M-16V does not have azoreductase or nitroreductase activity.
 - j. *B. breve* M-16V does not have hemolytic activity.
 - k. *B. breve* M-16V has no deleterious effects on platelet aggregation or viability.
 - l. *B. breve* M-16V has no mucolytic activity.
9. Toxicology studies show no evidence of *B. breve* M-16V translocating the gut epithelium, it does not induce mutations in *Salmonella typhimurium* strain TA98 and TA100 with or without metabolic activation, and does not produce test article-related toxicity in a 90-day repeated dose oral toxicity study in rats administered 2.3×10^{11} cfu *B. breve* M-16V/kg/day.
10. Twelve studies published from 1992 – 2012 have reported the safe administration of *B. breve* M-16V at doses up to 1.5×10^{10} cfu/d for durations of up to 3 months in a total of 430 health compromised and/or premature infants. *B. breve* M-16V was well tolerated and no treatment-related adverse health effects were noted.

11. *B. breve* M-16V strain has been administered in two studies in adults at a dose of 2×10^{10} cfu/d for 4-8 wk in patients with intermittent to mild persistent asthma or atopic dermatitis. There were no treatment-related adverse effects noted.
12. Other non-M-16V strains of *B. breve* have been administered to adults and children at doses up to 8×10^{11} cfu/d for durations up to 1 yr and no adverse treatment-related effects were reported.
13. Assuming a maximum addition of 5.0×10^9 cfu *B. breve* M-16V/serving of the conventional foods listed in this document, the estimated mean and 90th percentile 2-day average intakes of *B. breve* M-16V from all categories combined in the population ages 2 years and older are 3.8×10^{10} and 6.0×10^{10} cfu/day, respectively.
14. Under the most conservative assumptions, a maximum addition of 10^8 cfu of *B. breve* M-16V per gram of powdered medical foods containing hydrolyzed proteins and/or amino acid mixtures for children ages one to ten years old, the estimated daily intake of a one year-old and a ten year-old is 1.6×10^{10} cfu and 4.2×10^{10} cfu *B. breve* M-16V per day, respectively.

Determination of the GRAS status of *B. breve* M-16V under the intended conditions of use has been made through the deliberations of A. Wallace Hayes, PhD, DABT, FATS, ERT, CNS, FACN; Gregor Reid, PhD, MBA, ARM, CCM, Dr HS, FCAHS; Roger Clemens, Dr PH, CNS, FACN, FIFT. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of *B. breve* M-16V and the human exposure to *B. breve* M-16V resulting from its intended use as an ingredient in selected conventional foods and medical foods containing hydrolyzed proteins and/or amino acid mixtures for children, and have concluded:

There is no evidence in the available information on B. breve M-16V that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when B. breve M-16V is used at levels that might reasonably be expected from the proposed applications. B. breve M-16V is GRAS for use in selected conventional and medical foods as proposed by Morinaga Milk Industry Co., Ltd.

Therefore, *B. breve* M-16V is safe and GRAS at the proposed levels of addition to foods. *B. breve* M-16V is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

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E. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., President, Spherix Consulting, Incorporated, 6430 Rockledge Drive, Westmoreland Bldg, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ClaireK@chromadex.com.

F. SIGNATURE

Pursuant to the criteria provided in proposed 21 CFR 170.36, Morinaga Milk Industry Co., Ltd. hereby notifies the Food and Drug Administration that the use of *B. breve* M-16V in foods under the intended conditions of use is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Morinaga Milk Industry Co., Ltd. has determined that such use is Generally Recognized As Safe through scientific procedures.

(b) (6)



Signature
Claire L. Kruger, Authorized Representative of
Morinaga Milk Industry Co., Ltd.

December 14, 2012

Date

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II. DESCRIPTION OF SUBSTANCE

A. COMMON OR USUAL NAME

Bifidobacterium breve M-16V

B. SYSTEMATIC IDENTIFICATION

B. breve M-16V has been deposited in the Belgian Co-ordinated Collections of Microorganisms (BCCM) and has been designated LMG 23729. Other species names include *B. breve* Reuter 1963 AL, Mitsuoka 16, PRSF-B105, BB-576, *biovar a*, YY, and Yaeshima M-16V.

C. CLASSIFICATION

1. Source

B. breve M-16V was isolated from the feces of a healthy infant in 1963.

2. Morphology

B. breve M-16V is a non-motile, non-spore forming, rod-shaped anaerobic Gram-positive bacterium.

3. Genotypic Identification

The genome of *B. breve* M-16V was sequenced and assembled into a single chromosome consisting of 2,269,379 base pairs. Comparative analysis of guanine and cytosine content (Table 1) and genome-wide homology analysis using a plate-based form of Southern blotting (Table 2) showed that *B. breve* M-16V is genetically most similar to the type strain *B. breve* ATCC 15700 and dissimilar to other strains of bifidobacteria. Basic Local Alignment Search Tool for Nucleotides (BLASTN) analyses of the 16S rDNA sequences of *B. breve* M-16V and *B. breve* ATCC 15700 also revealed high amounts of homology between the two strains (Appendix 1). The methods used for the genotypic identification are described in Appendix 4.

BOX-A1R-based repetitive extragenic palindromic-polymerase chain reaction (BOX-PCR) fingerprinting and cluster analysis performed by PROSAFE [Biosafety Assessment of Probiotics used for Human Consumption; Appendix 2A and 2B (*B. breve* M-16V referred to as Original Number EU-PS38 in Appendix 2B)], 16S rDNA sequencing performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Appendix 2C, *B. breve* M-16V referred to as strain NR1 (ID 05-1030)), and fluorescent amplified fragment length polymorphism (FAFLP) performed by BCCM/LMG (Appendix 2D; *B. breve* M-16V referred to NumRES TD1 or ID9054) confirmed that *B. breve* M-16V is a member of *B. breve*.

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Test Species	Guanine and Cytosine content (mole %)
<i>Bifidobacterium breve</i> M-16V	58.9
<i>Bifidobacterium breve</i> ATCC 15700	58.9
<i>Escherichia Coli</i> Strain number	52.9

¹GC content was determined by thermal melting point analysis as described in Appendix 4.

Test Species	% DNA Similarity to <i>B. breve</i> strains	
	<i>B. breve</i> M-16V	<i>B. breve</i> ATCC 15700
<i>B. breve</i> M-16V	100.0	94.2
<i>B. breve</i> ATCC 15700 ^b	93.4	100.0
<i>Bifidobacterium infantis</i> ATCC 15697 ^b	46.5	55.6
<i>Bifidobacterium bifidum</i> ATCC 29521 ^b	17.3	29.8
<i>Bifidobacterium pseudolongum</i> subsp. <i>pseudolongum</i> ATCC 25526 ^b	19.6	22.4
<i>Bifidobacterium pseudolongum</i> subsp. <i>globosum</i> ATCC 25865 ^b	15.3	17.1
<i>Bifidobacterium longum</i> ATCC 15707 ^b	53.9	56.9

^aGenomic homology was determined using a modified form of Southern blotting as described in Appendix 4.
^bPurchased from the American Type Culture Collection (ATCC).

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4. Phenotypic Identification

a. Carbohydrate Fermentation Pattern

The carbohydrate fermentation pattern (CFP) and fructose 6-phosphate phosphoketolase (F6PPK) activity of *B. breve* M-16V was compared to that of *B. breve* ATCC 15700 as described (Boyd et al., 2005; Scardovi, 1986). The CFPs were qualitatively similar (Table 3) and both strains have F6PPK activity, indicating that *B. breve* M-16V is phenotypically similar to *B. breve* ATCC 15700. DSMZ also determined the CFP for *B. breve* M-16V and found similar results (Appendix 2C, *B. breve* M-16V referred to as strain NR1 (ID 05-1030)). An analysis of the fermentation products produced by *B. breve* M-16V when grown with different carbon sources showed that *B. breve* M-16V produces mainly acetic acid and L-lactic acid (Figure 1). The methods used to quantify these products are described in Appendix 4.

Table 3. Carbohydrate Fermentation Pattern of <i>Bifidobacterium breve</i> M-16V		
Carbon source	Strain	
	<i>B. breve</i> M-16V	<i>B. breve</i> ATCC 15700
Glycerol	-	-
Erythritol	-	-
D-arabinose	(+)S	(+)
L-arabinose	-	-
Ribose	+	+
D-xylose	-	-
L-xylose	-	-
Adonitol	-	-
β-methyl-xyloside	-	-
Galactose	+	+
D-glucose	+	+
D-fructose	+	+
D-mannose	+	+
L-sorbose	-	-
Rhamnose	-	-
Dulcitol	-	-
Inositol	-	-
Mannitol	+	+
Sorbitol	+	+
α-methyl-D-mannoside	-	-
α-methyl-D-glucoside	+	+
N-acetyl glucosamine	-	(+)

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Table 3. Carbohydrate Fermentation Pattern of <i>Bifidobacterium breve</i> M-16V		
Carbon source	Strain	
	<i>B. breve</i> M-16V	<i>B. breve</i> ATCC 15700
Amygdalin	+	+
Arbutin	+	+
Esculin	+	+
Salicin	+	+
Cellobiose	+	+
Maltose	+	+
Lactose	+	+
Melibiose	+	+
Sucrose	+	+
Trehalose	-	-
Inulin	-	-
Melezitose	-	-
Raffinose	+	+
Starch	+	+S
Glycogen	+	+
Xylitol	-	-
Gentiobiose	+S	+S
D-turanose	+	+
D-lyxose	-	-
D-tagatose	-	-
D-fucose	-	-
L-fucose	+	+
D-arabitol	-	-
L-arabitol	-	-
Gluconate	-	-
2 keto-gluconate	-	-
5 keto-gluconate	-	±S

Notes:

1. *B. breve* M-16V and *B. breve* ATCC 15700 were cultured as described (Matsuki et al., 1999).
2. “-“ negative; “±” very weakly positive; “(+)” weakly positive; “+” positive; “S” delayed reaction.

000025

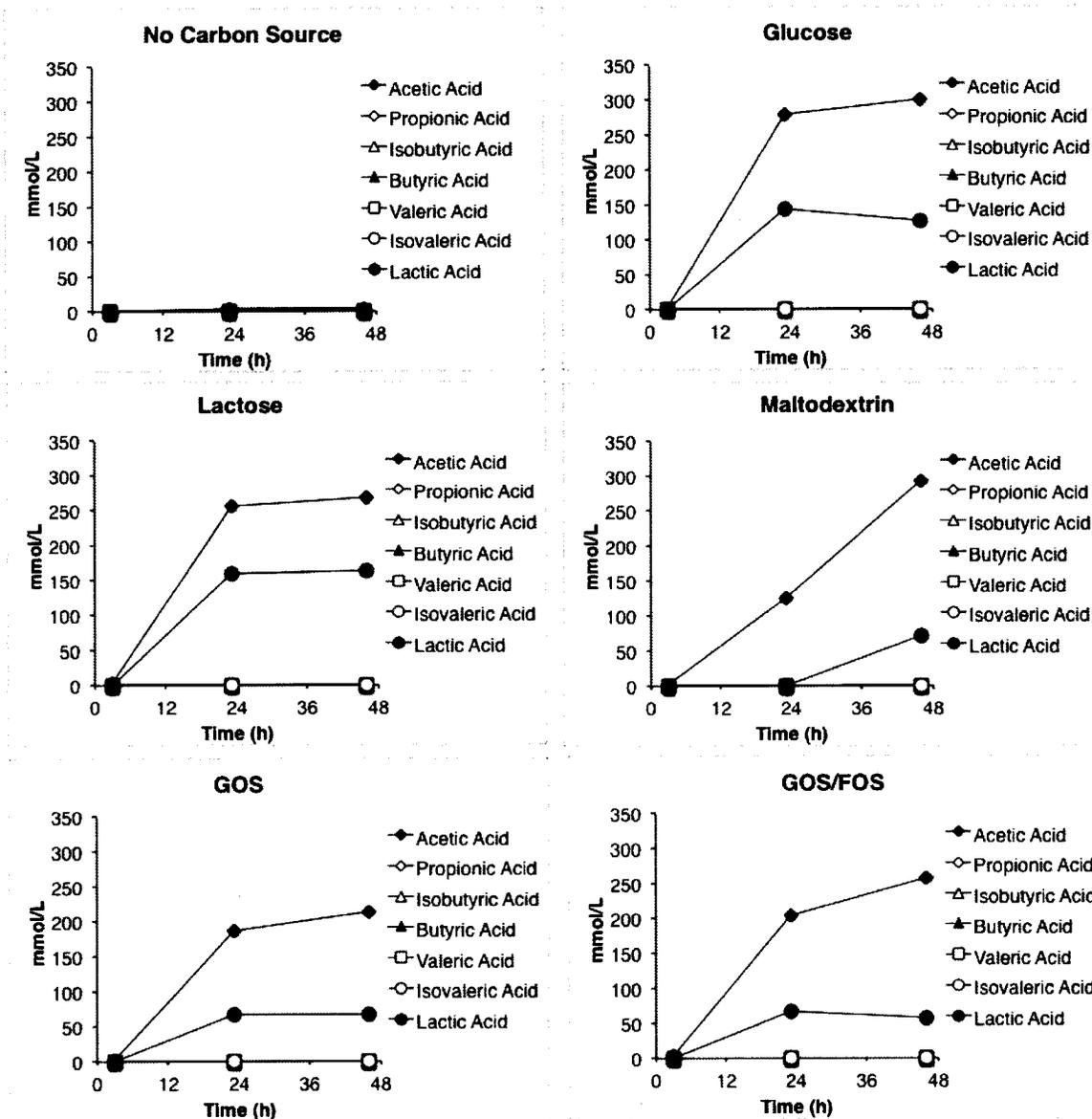


Figure 1. *Bifidobacterium breve* M-16V is a lactic acid- and acetic acid-producing bacterium.

B. breve M-16V was grown in medium containing glucose, lactose, maltodextrin, galacto-oligosaccharide (GOS) and galacto-oligosaccharide/fructo-oligosaccharide (GOS/FOS) and fermentation product were determined using enzymatic kits and gas chromatography as described in Appendix 4.

b. Resistance to Bile Acids

Bile acids may prevent bacterial overgrowth in the small intestine (Lorenzo-Zuniga et al., 2003; Ding et al., 1993) and it is generally believed that probiotic bacteria are able to survive their passage through the gastrointestinal tract. To determine whether or not *B. breve* M-16V was able to persist in the presence of bile acids, *B. breve* M-16V and the type strain *B. breve* ATCC 15700 were incubated in medium containing increasing amounts of porcine bile extract and their growth was compared (Figure 2). The presence of 0.1 % and 0.3 % bile extract had no effect on the growth of *B. breve* M-16V and *B. breve* ATCC 15700 up to 6 h. After 24 h, the growth of *B. breve* M-16V was reduced in the medium containing 0.1 % bile extract and markedly reduced in the medium containing 0.3% bile extract. The growth of *B. breve* ATCC 15700, the type strain, was unaffected in the medium containing 0.1 % bile extract and greatly reduced in the medium containing 0.3% bile extract. These results suggest that *B. breve* M-16V is similar to the type strain and may be able to survive during its passage through the human digestive tract. The methods used to determine the resistance of *B. breve* M-16V to bile acids are described in Appendix 4.

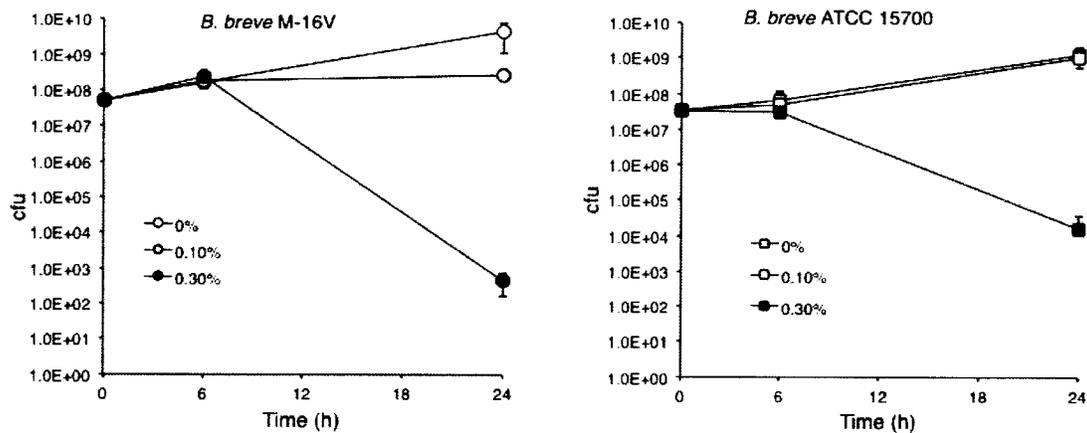


Figure 2. Growth of *Bifidobacterium breve* M-16V in medium containing increasing amounts of a porcine bile extract.

***B. breve* M-16V and *B. breve* ATCC 15700 were cultured in the presence of increasing amounts of porcine bile extract and the number colony forming units (cfu) were compared at different times over a 24 hr growth period (Appendix 4).**

D. PRODUCTION PROCESS

The production of commercial *B. breve* M-16V consists of a culturing process and a non-culturing process (Figure 3), which have been certified as meeting the requirements of Hazard Analysis and Critical Control Point (HACCP) Codex Alimentarius (Appendix 3) and are typical for the production of probiotics used in food. The culturing process is a series of sequential expansions of working stocks, which are derived from the original stocks of *B. breve* M-16V,

yielding a manufacturing culture. The manufacturing culture provides the material for the non-culturing process, which refines and prepares *B. breve* M-16V for distribution.

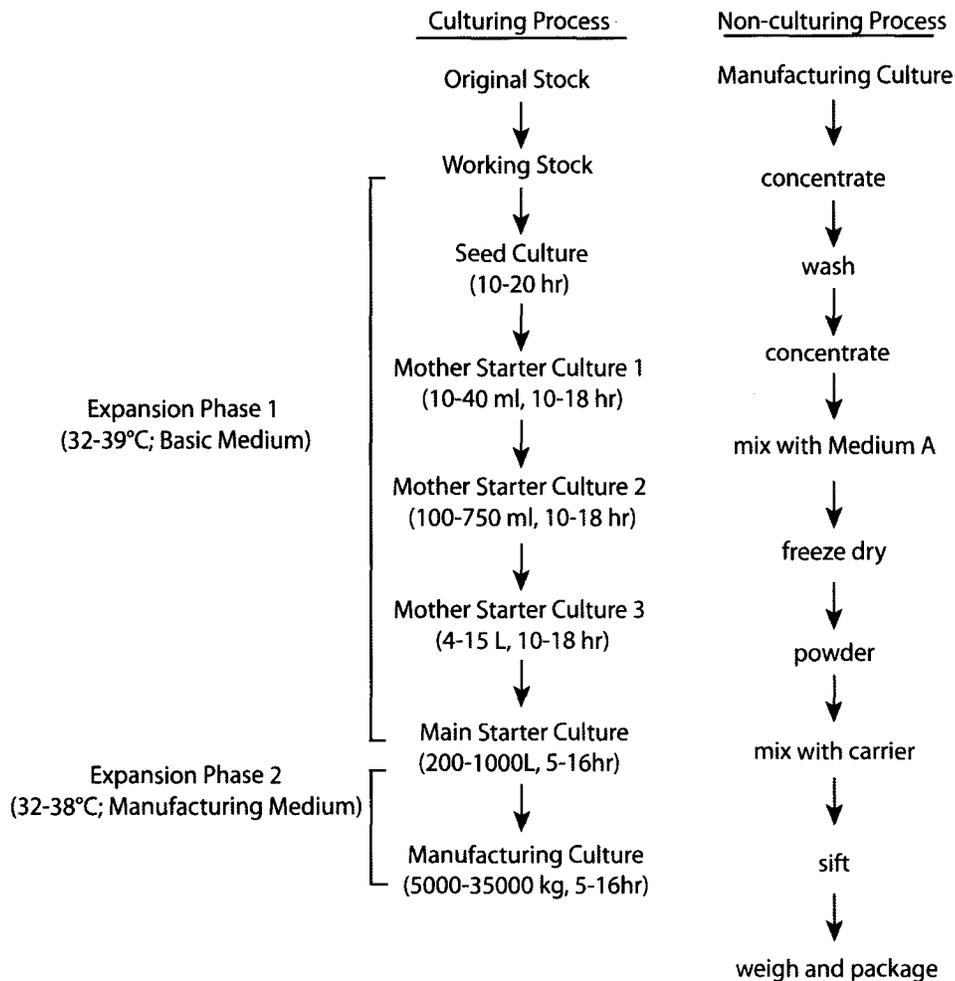


Figure 3. Production process of commercial *Bifidobacterium breve* M-16V. Original and working stocks are maintained in the Research and Development Center at the Morinaga Milk Industry. For the production of finished products, frozen working stocks are transferred to the Morinaga Tone Factory in the Ibaraki Prefecture for their expansion and preparation of distribution.

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1. **Culturing Process**

a. *Original Stocks*

B. breve M-16V was isolated from the feces of a healthy infant in 1963 and identified as a strain of *B. breve* by morphologic, genetic, and phenotypic analyses (described in section II.C). Original stocks of *B. breve* M-16V are stored at -80°C and maintained by the Biological Function Research Department of the Food Science and Technology Institute at the Morinaga Research and Development Center. The strain has also been deposited at BCCM.

b. *Working Stocks*

Working stocks are established by thawing and expanding the original stock and freezing 10-fold dilutions at -80°C. The vials are then stored at the Morinaga Laboratory. The frozen working stocks are transferred to the Morinaga Tone Factory in the Ibaraki Prefecture, Japan, thawed and grown to generate seed cultures, which provide the starting material for expansion Phase 1.

c. *Expansion Phase 1*

Seed cultures are sequentially expanded as follows in mother starting culture 1, mother starting culture 2, mother starting culture 3, and main starter culture.

d. *Expansion Phase 2*

The main starter culture is expanded a final time to generate the manufacturing culture.

2. **Non-culturing Process**

The manufacturing culture is cooled, concentrated, washed, reconcentrated, resuspended, freeze-dried, crushed to a powder, and mixed with powdered carbohydrate carriers, which are either cornstarch, tapioca starch or dextrin. The *B. breve* M-16V/carbohydrate mix is then sifted, passed through a magnetic tunnel to remove contaminating metal particles, weighed, packed into airtight bags, and stored at 10°C.

E. **QUALITY CONTROL**

Morinaga Milk Industry routinely evaluates the quality of the *B. breve* M-16V products during the production process to ensure that the genetic identity is consistent with that of the original stock and the finished products are free of contaminants. The timing and parameters measured during the culturing and non-culturing processes are provided in Table 4.

Table 4. Quality Control Parameters Monitored During the Production Process of *Bifidobacterium breve* M-16V²

Parameter ²	Culturing Process					Non-culturing Process			
	Expansion Phase 1					Expansion Phase 2			
	Seed Culture	Mother Starter Culture 1	Mother Starter Culture 2	Mother Starter Culture 3	Main Starter Culture	Manufacturing Culture	Mixing with Medium A	Powdering	Finished Product
Culture pH	X ¹	X	X	X	X	X	X		
Cell morphology	X	X	X	X	X	X	X		
Anaerobic cfu						X	X	X	X
Aerobic cfu						X	X	X	X
Mold							X	X	X
Yeast							X	X	X
Coliform (including <i>E. coli</i>)							X	X	X
<i>Staphylococcus aureus</i>							X	X	X
<i>Salmonella</i>							X	X	X
<i>Cronobacter sakazakii</i>							X	X	X
<i>Clostridium perfringens</i>									X
<i>Bacillus cereus</i>									X
Enterobacteriaceae									X
Enterococci									X
Loss on Drying								X	X
Arsenic									X
Other heavy metals									X
Lead									X
Casein									X
β-Lactoglobulin									X
Appearance									X
Foreign body							X	X	X
Odor and taste									X
Radioactivity									X
RAPD-PCR									X

¹"X" denotes that the parameter is measured.

²All methods used to determine each parameter are described in Appendix 4.

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F. FINISHED PRODUCT DESCRIPTIONS AND SPECIFICATIONS

Morinaga Milk Industry produces two *B. breve* M-16V-containing products, M-16V and M-16V Type T. M-16V is mixed with cornstarch and M-16V Type T is mixed with tapioca starch or dextrin. Both contain approximately 10^{11} cfu *B. breve* M-16V/g.

1. Genotypic Specifications and Batch Records

The genotype of *B. breve* M-16V in M-16V and M-16V Type T is routinely evaluated for each batch by comparing the electrophoretic banding patterns of random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR) products amplified from DNA obtained from the original stock and lots of M-16V as described in Appendix 4. An example of the RAPD-PCR analysis of three different lots of M-16V and M-16V Type T is provided in Figure 4.

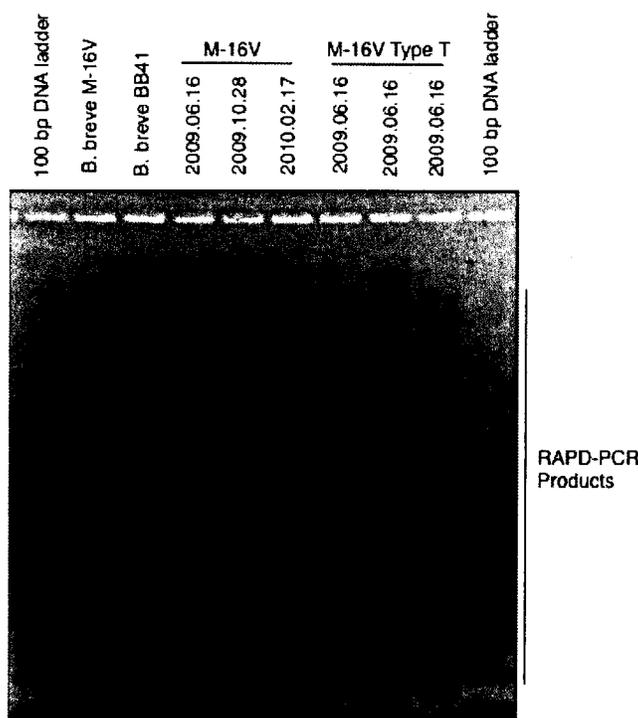


Figure 4. RAPD-PCR analysis of three lots of M-16V and M-16V Type T finished products. DNA was obtained from the *Bifidobacterium breve* (*B. breve*) M-16V culture, *B. breve* BB41, three lots of *B. breve* M-16V, and three lots of *B. breve* M-16V Type T, and amplified by RAPD-PCR using RP1 primers as described in Appendix 4.

2. Physical Specifications and Batch Records

Each batch of M-16V and M-16V Type T is evaluated by Moringa Milk Industry against specifications described in Table 5 and Table 6, and the quality of three lots of M-16V and M-16V Type T products have also been included in Table 5 and Table 6, respectively. The supporting documents are provided in Appendix 5A and 5B.

Table 5. Product Specifications and Lot Data for M-16V

Parameter	Method	Specifications	Lot No. (Indicates Production Date)		
			2009.06.16	2009.10.26	2010.07.17
Physical Characteristics					
Appearance	Visual inspection	White to slightly brown powder	White to slightly brown powder	White to slightly brown powder	White to slightly brown powder
Foreign body	Visual inspection	Negative	Negative	Negative	Negative
Odor and Taste	Sensory evaluation	No abnormal odor and taste	No abnormal odor and taste	No abnormal odor and taste	No abnormal odor and taste
Microbiological Characteristics					
Anaerobic cfu ¹ (<i>Bifidobacterium breve</i> M-16V)	Reinforced Clostridial Agar ²	> 1.0 x 10 ¹¹ cfu/g	2.3 x 10 ¹¹	2.3 x 10 ¹¹	2.0 x 10 ¹¹
Aerobic cfu	Standard Plate Count Agar ²	< 300 cfu/g	< 300(0)	< 300(0)	< 300(0)
Enterobacteriaceae	EE and VRBD ²	Negative/0.01 g	Negative	Negative	Negative
<i>Escherichia coli</i>	EE and ECB ²	Negative/1 g	Negative	Negative	Negative
Enterococci	Azide Citrate Medium ²	Negative/0.01 g	Negative	Negative	Negative
<i>Clostridium perfringens</i>	Clostridia Count Agar ²	Negative/ 0.1 g	Negative	Negative	Negative
<i>Staphylococcus aureus</i>	Mannitol Salt Agar with egg yolk ²	Negative/0.01 g	Negative	Negative	Negative
Mold	Potato Dextrose Agar ²	< 30 cfu/g	< 10(0)	< 10(0)	< 10(0)
Yeast	Potato Dextrose Agar ²	< 30 cfu/g	< 10(0)	< 10(0)	< 10(0)
<i>Salmonella</i>	BPW/DHL Agar ²	Negative/25 g	Negative	Negative	Negative
<i>Cronobacter sakazakii</i>	BPW/EE/VRBD Agar ²	Negative/25 g	Negative	Negative	Negative
<i>Bacillus cereus</i>	NGKG Agar ²	< 100 cfu/1 g	< 30(0)	< 30(0)	< 30(0)
Metals					
Lead	ICP-MS	< 0.2 ppm	< 0.2	< 0.2	< 0.2
Arsenic	X-ray Fluorescence	< 1 ppm	< 1	< 1	< 1
Other heavy metals	X-ray Fluorescence	< 5 ppm	< 5	< 5	< 5
Other					
Loss on drying	Drying at 105°C for 4 h	< 6 g/100 g	0.6	1.7	1.9
Casein	ELISA	< 0.1 ppm	< 0.1	< 0.1	< 0.1
β-Lactoglobulin	ELISA	< 0.1 ppm	< 0.1	< 0.1	< 0.1
¹ “cfu” denotes colony-forming units. ² The amount of <i>Bifidobacterium breve</i> M-16V and microbiological contaminants in finished products is determined by their growth on selective media. The analytical methods and media used are described in Appendix 4. ³ (0) indicates that no colonies were detected at the minimum dilution.					

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Table 6. Product Specifications and Lot Data for M-16V Type T

Parameter	Method	Specifications	Lot No. (Indicates Production Date)		
			2006-04-28	2008-09-22	2011-06-21
Physical Characteristics					
Appearance	Visual inspection	White to slightly brown powder	White to slightly brown powder	White to slightly brown powder	White to slightly brown powder
Foreign body	Visual inspection	Negative	Negative	Negative	Negative
Odor and Taste	Sensory evaluation	No abnormal odor and taste	No abnormal odor and taste	No abnormal odor and taste	No abnormal odor and taste
Microbiological Characteristics					
Anaerobic cfu ¹ (<i>Bifidobacterium breve</i> M-16V)	Reinforced Clostridial Agar ²	> 1.0 x 10 ¹¹ cfu/g	2.1 x 10 ¹¹	1.8 x 10 ¹¹	2.5 x 10 ¹¹
Aerobic cfu	Standard Plate Count Agar ²	< 300 cfu/g	< 300(0)	< 300(0)	< 300(0)
Coliform bacteria	BGLB broth ²	Negative/1 g	Negative	Negative	Negative
<i>Staphylococcus aureus</i>	Mannitol Salt Agar with egg ² yolk	Negative/0.01 g	Negative	Negative	Negative
Mold	Potato Dextrose Agar ²	< 30 cfu/g	< 10(0)	< 10(0)	< 10(0)
Yeast	Potato Dextrose Agar ²	< 30 cfu/g	< 10(0)	< 10(0)	< 10(0)
<i>Salmonella</i>	BPW/DHL Agar ²	Negative/5 g	Negative	Negative	Negative
Metals					
Arsenic	X-ray Fluorescence	< 1 ppm	< 1	< 1	< 1
Other heavy metals	X-ray Fluorescence	< 5 ppm	< 5	< 5	< 5
Other					
Loss on drying	Drying at 105°C for 4 h	< 6 g/100 g	0.8	1.0	0.7
Casein	ELISA	< 0.1 ppm	< 0.1	< 0.1	< 0.1
β-Lactoglobulin	ELISA	< 0.1 ppm	< 0.1	< 0.1	< 0.1
¹ “cfu” denotes colony-forming units. ² The amount of <i>Bifidobacterium breve</i> M-16V and microbiological contaminants in finished products is determined by their growth on selective media. The analytical methods and media used are described in Appendix 4. ³ (0) indicates that no colonies were detected at the minimum dilution.					

3. Allergenic Proteins

Finished products containing *B. breve* M-16V are free of soy, egg, peanuts, treenuts, wheat, shellfish, milk, and fish products. To confirm that no cross-contamination with milk products occurred during manufacture, Morinaga analyzes each batch of finished product to ensure that they contain less than 0.1 ppm of casein and β -lactoglobulin. The methods used to determine casein and β -lactoglobulin concentrations are described in Appendix 4.

4. Survivability of Finished Product

The survivability of *B. breve* M-16V in M-16V and M-16V Type T in finished product was determined as described by Abe et al. (2009a). Both products were stored at different temperatures for increasing amounts of time and the number of viable *B. breve* M-16V was determined by anaerobically culturing reconstituted samples from the stored products on selective medium and counting the number of colonies (Appendix 4). Storage of M-16V and M-16V Type T at 5 and 25°C caused similar albeit minimal losses in *B. breve* M-16V viability over the 36 month storage period (Figure 5). Storage of both products at 37°C decreased the viability of *B. breve* M-16V, to a greater extent than storage at 5 and 25°C. At the end of shelf-life, the viability of all products at all temperatures (5°, 25° and 37°C) was approximately 10^{11} cfu/g or greater.

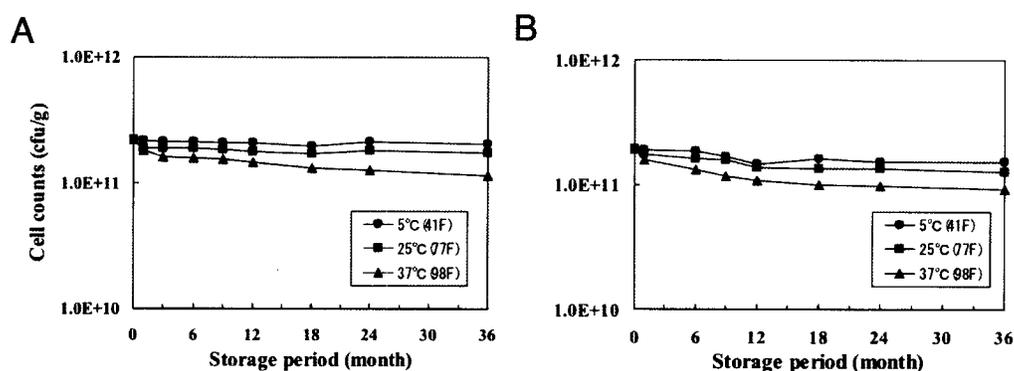


Figure 5. Viability of *Bifidobacterium breve* M-16V in M-16V (A) and M-16V Type T (B) finished products.

The products were incubated at 5, 25, and 37°C for increasing amounts of time and the number of viable *B. breve* M-16V was determined at each time point by anaerobically culturing a reconstituted sample on selective medium and counting the number of colonies (Appendix 4).

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G. MARKET PRODUCTS

Akachan-no Bifidus is a powdered food currently marketed in Japan that contain *B. breve* M-16V. To evaluate the survivability of *B. breve* M-16V, the products were stored at 5, 25, and 37°C for 24 months and viability was determined at each time point by anaerobically culturing a reconstituted sample on selective medium and counting the number colonies (Appendix 4). Storage of Akachan-no Bifidus at 5 and 25°C did not significantly affect the viability of *B. breve* M-16V over the course of the 24 months. Storage at 37°C decreased *B. breve* M-16V viability to approximately 10^{10} cfu/g by 3 months and remained at approximately 10^{10} cfu/g throughout the remainder of the testing period (Figure 6).

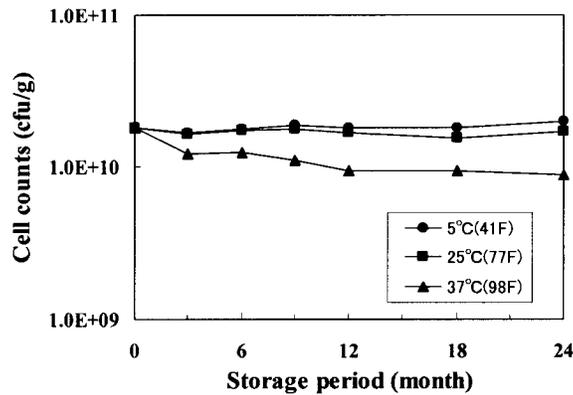


Figure 6. Stability of *Bifidobacterium breve* M-16V in Akachan-no Bifidus.
The product was incubated at 5, 25, and 37°C for increasing amounts of time, and the number of viable *B. breve* M-16V was determined at each time point by anaerobically culturing a reconstituted sample on selective medium and counting the number of colonies (Appendix 4).

Infant formula for cow's milk allergic infants is prototype powdered product being developed by Morinaga Milk Industry Co., Ltd. and storage of this product at 5 and 25°C also did not significantly effect *B. breve* M-16V viability. However, storage at 37°C decreased *B. breve* M-16V viability in a time-dependent fashion, resulting in less than 10^7 cfu/g at 24 months (Figure 7).

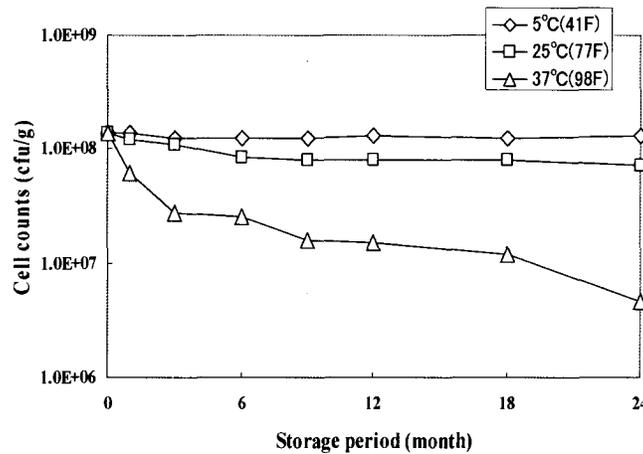


Figure 7. Stability of *Bifidobacterium breve* M-16V in powdered formula for cow's milk allergic infants.

The product was incubated at 5, 25, and 37°C for increasing amounts of time, and the number of viable *B. breve* M-16V was determined at each time point by anaerobically culturing a reconstituted sample on selective medium and counting the number of colonies (Appendix 4).

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III. INTENDED EFFECT

Bifidobacteria are normal inhabitants of the human gastrointestinal tract and are commonly used as probiotics. Their consumption has been associated with a variety of beneficial effects both systemically and in the gastrointestinal tract (reviewed in Russell et al., 2011; Naidu et al., 1999). Furthermore, bifidobacteria supplemented in the diet do not appear to colonize the gastrointestinal tract (Kullen et al., 1997; Grmanová et al., 2010).

The intended effect is to provide a dietary source of *B. breve* M-16V to conventional foods and medical foods.

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IV. HISTORY OF USE

A. HISTORICAL EXPOSURE TO *B. breve* M-16V

Bifidobacteria have been consumed in fermented foods for decades and currently used commercial strains include *Bifidobacterium animalis* ssp. *lactis* strain Bf-6, *Bifidobacterium lactis* Bb-12, *Bifidobacterium lactis* DR10 (HN019), *Bifidobacterium longum* BB536, *Bifidobacterium breve* Yakult, *Bifidobacterium breve* SBT-2928, and *Bifidobacterium breve* C50. In the United States *B. animalis* ssp. *lactis* Bf-6 has been approved for use in conventional foods (GRN 377), *B. lactis* Bb-12 has been approved for use in formulas for infants four months of age and older (GRN 49), and *B. longum* BB536 has been approved for use in selected foods (GRN 268). Other probiotics, such as *Lactobacillus reuteri* DSM 17939, have been approved for use in term infant formulas (GRN 410). Furthermore, there is no evidence to date showing that the consumption of non-viable bifidobacteria in fermented foods is unsafe.

Morinaga Milk Industry is the sole proprietor of *B. breve* M-16V and introduced the bacterium into the Japanese market in 1976 in a dietary supplement called Vinelac. In 1982, Morinaga added *B. breve* M-16V to a growing-up powdered formula called Yochien-Jidai and has since added it to several other products (Table 7).

Date	Product	Market	Total Tons Sold (Years)
1976	Vinelac	Japan	Unavailable
1982	Yochien-Jidai (growing-up formula)	Japan	Unavailable
2004	Akachan-no Bifidus	Japan	0.41 ^b (2006 – 2011)
2006	Growing-up milk formula ^c	Indonesia	9919 (2006 - 2011)
2009	Follow-on milk formula ^d	Indonesia	2215 (2009 - 2011)

^aProvided by Morinaga Milk Industry Co., Ltd.
^bTotal tons of Akachan-no Bifidus sold is not accurate because the product is currently sold via the company website only.
^cGrowing-up milk formula is intended for infants one year and older.
^dFollow-on milk formula is intended for infants 7 to 11 months-old.

B. INTENDED USES AND ESTIMATED INTAKES OF *B. breve* M-16V

1. Intended Uses

Morinaga Milk Industry Co., Ltd. intends to add *B. breve* M-16V to selected food products for the general population (Table 8), and formulas and products containing hydrolyzed proteins and/or amino acid mixtures for children. Selected foods will contain 5×10^9 cfu/serving, powdered formulations will contain 10^8 cfu/g, and the products containing hydrolyzed proteins and/or amino acid mixtures for children will contain only M-16V finished products. Morinaga Milk Industry will ensure the stability and viability of *B. breve* M-16V so that the products deliver the declared levels in a serving of stated size throughout the product shelf life.

Table 8. Proposed Conventional Food Categories for the Addition of <i>Bifidobacterium breve</i> M-16V	
Food Category	Specific Food Type
Breads/baked goods	<ol style="list-style-type: none"> 1. Bars; includes meal replacement, high protein, snack bars 2. Biscuits 3. Breads/roll (yeast), including bagels, croissants, English muffins, pizza crust 4. Breakfast pastries; includes Danish 5. Cakes, includes coffee cakes 6. Cobblers, turnovers, strudels, crisps 7. Cookie bars 8. Crackers 9. Doughnuts 10. Pies 11. Quick breads; includes breads, muffins, popovers, cornbread
Cereals	<ol style="list-style-type: none"> 1. Breakfast cereals, cooked; includes grits, oatmeal, cream of wheat, and wheat cereal 2. Breakfast cereals, ready-to-eat
Fruits	Juices and nectars, including citrus, non-citrus, vegetable and blends, frozen fruit, frozen juice bars, ices

Table 8. Proposed Conventional Food Categories for the Addition of *Bifidobacterium breve* M-16V

Food Category	Specific Food Type
<p>Dairy products/dairy-based foods and dairy substitutes</p>	<ol style="list-style-type: none"> 1. Skim milk 2. Cheese spreads 3. Cheese, imitation 4. Cheese, processed 5. Cream substitutes 6. Cream, heavy 7. Fermented milk (flavored, heat treated), including buttermilk, kefir, and flavored milk beverage mixes 8. Frozen desserts, including ice cream, ice milk, frozen yogurt, frozen novelties, and imitation milk 9. Meal replacements, liquids and dry mixes 10. Milk shakes 11. Milk (plain and flavored), including cocoa, chocolate milk, fruit milks, coffee drinks (fluid/dry) 12. Puddings and custards 13. Smoothies 14. Whipped toppings 15. Yogurt 16. Butter and dried milk products 17. Milk powder for pregnant women, plain and flavored 18. Milk powder for adult people, plain and flavored 19. Milk powder for elderly people, plain and flavored
<p>Miscellaneous</p>	<ol style="list-style-type: none"> 1. Candies, including hard candies, mints, chocolate and all other types of confections (i.e., chewing gum), cocoa powder, condiment sauces, (i.e., catsup, BBQ, taco, steak, cocktail, Worcestershire, teriyaki, cheese-based, hollandaise, tartar, béarnaise) 2. Gelatin desserts, plain or with fruit gravies 3. Peanut and other nut butters/spreads 4. Snack foods, including chips, popcorn mixtures 5. Weaning foods, including meals, desserts, fruits, cereal, vegetables, meat sticks, snacks, juices

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2. Estimated Intakes of *B. breve* M-16V

a. Conventional Foods

Estimates of potential intake of *B. breve* M-16V resulting from the intended uses of *B. breve* M-16V in conventional foods were calculated using food consumption data reported in the United States Department of Health and Human Service's 2003-2004 National Health and Nutrition Examination Survey (NHANES), the proposed used level of 5.0×10^9 cfu/serving, and serving sizes reported in 21 CFR 101.12.

The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States (NCHS 2006). As part of the survey, trained dietary interviewers collect detailed information on all foods and beverages consumed by respondents in the previous 24 hour time period (midnight to midnight). A second dietary recall was administered by telephone 3 to 10 days after the first dietary interview, but not on the same day of the week as the first interview. A total of 9034 respondents provided complete dietary intakes for the Day 1 recall, and 8354 of the individuals provided a complete Day 2 recall.

The data files used to process the NHANES 2003-2004 dietary recalls include 6940 food codes, each identified by a unique number and descriptive name. The food codes represent single component foods or ingredients such as milk or vegetable oil; finished foods such as bread or margarine; and also food mixtures such as a grilled cheese sandwich. The database was reviewed, and all food codes corresponding to one of the intended use categories of *B. breve* M-16V were identified. USDA survey files (USDA 2006) were used to identify the proportion of food mixtures represented by applicable use categories. For example, food codes for "cheeseburger" may be included in estimates of the bread/roll, processed cheese, and catsup use categories based on the percentage (by weight) of each component in the USDA files corresponding to the "cheeseburger" food codes.

Using the list of food codes and the NHANES 2003-2004 dietary recall data files from individuals with two complete days of dietary recall, Spherix estimated mean and 90th percentile 2-day average intakes of the individual proposed product categories and also all categories combined. Two-day average intakes represent the total number of servings consumed during the two days of recall divided by two (i.e., $(\text{Intake}_{\text{Day 1}} + \text{Intake}_{\text{Day 2}})/2$). Intakes were calculated for subpopulations of children (2-5 y M+F, 6-11 y M and F separately), teenagers (12-18 y M and F separately) and adults (19+ y M and F separately)(Table 9). The estimates were generated using

survey sample weights to adjust for differences in representation of subpopulations; results therefore are representative of the U.S. population. The population ages 2 years and older consumed a mean of 7.5 servings of the target foods per day, while the 90th percentile of intake was 12.5 servings per day. Among the subpopulations, mean 2-day average intakes from all categories combined ranged from 6.6 to 9.3 servings per day, and 2-day average 90th percentile intakes ranged from 10.6 to 15.3 servings per day.

Assuming a maximum addition of 5.0×10^9 cfu *B. breve* M-16V/serving of the target food categories, the estimated mean and 90th percentile 2-day average intakes of *B. breve* M-16V from all categories combined in the population ages 2 years and older are 3.8×10^{10} and 6.0×10^{10} cfu/day, respectively (Table 9). Across all of the subpopulations, the maximum estimated daily average 90th percentile intake of *B. breve* M-16V from all categories combined is approximately 8.0×10^{10} cfu for males ages 12-18 years. Importantly, all the estimates presented in Table 8 are conservative because the viability of *B. breve* M-16V in market products decreases as the shelf-life increases (Figure 6 and 7), consumers may only consume a subset of different foods in two days, and some food processes may reduce the viable count of *B. breve* M-16V.

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Table 9. Estimated 2-Day Average Intakes of *Bifidobacterium breve* M-16V from Proposed Uses in Conventional Foods

Population (years)	Users (n)	Total Survey Sample	% Users ^b	Food (servings/d)		<i>B. breve</i> M-16V (cfu/d) ^a	
				Mean	90th percentile	Mean	90th percentile
Children 2-5	694	694	100	7.8	11.4	3.9 x 10 ¹⁰	5.0 x 10 ¹⁰
Males, 6-11	386	386	100	9.3	13.2	4.7 x 10 ¹⁰	7.0 x 10 ¹⁰
Females, 6-11	443	443	100	7.8	11.6	3.9 x 10 ¹⁰	6.0 x 10 ¹⁰
Males, 12-18	894	894	100	9.2	15.3	4.6 x 10 ¹⁰	8.0 x 10 ¹⁰
Females, 12-18	874	874	100	7.3	11.6	3.7 x 10 ¹⁰	6.0 x 10 ¹⁰
Males, 19+	2063	2064	100	8.0	13.1	4.0 x 10 ¹⁰	7.0 x 10 ¹⁰
Females, 19+	2297	2297	100	6.6	10.6	3.3 x 10 ¹⁰	6.0 x 10 ¹⁰
Total population,	7651	7652	100	7.5	12.5	3.8 x 10 ¹⁰	6.0 x 10 ¹⁰

^a Assumed a use level of 5 x 10⁹ cfu *B. breve* M-16V in each proposed use category (Table 7).
^b Weighted percent.

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b. Formulas Intended For Allergic Children From One To Ten Years-Old

Powdered formulas intended for allergic children from one to ten years-old, which may be used to supplement a restricted diet or in certain cases used as the sole source of nutrition, will contain no more than 10^8 cfu *B. breve* M-16V/g to produce an intended level of intake of 10^9 - 10^{10} cfu of *B. breve* M-16V/day. Under the most conservative assumptions that the selected food products are the sole source of nutrition, reconstituted at 20.9 g/100 ml and a caloric density of 1 kcal/ml, the standard dilution for enteral formulas for children over 12 months-old, and the caloric requirement of a one year-old and an active ten year-old is approximately 800 and 2000 kcal/day, respectively (Institute of Medicine (US) Panel on Macronutrients and Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 2005), the addition of 10^8 cfu *B. breve* M-16V/g of powdered formula will result in an intake of 1.6×10^{10} and 4.2×10^{10} cfu *B. breve* M-16V/day. Furthermore, the formulas will be supplemented appropriately to provide a minimum of 10^9 cfu *B. breve* M-16V/day at the end of an 18-month shelf-life at room temperature.

V. SAFETY OF *B. breve* M-16V

A. REVIEW OF BIFIDOBACTERIA

Bifidobacteria were discovered in the feces of infants in 1899 (Tissier, 1899; Tissier, 1900). Since then bifidobacteria have been found in a variety of ecological niches, including the oral cavity and vagina of humans, and multiple species and variants have been identified (Korshonov et al., 1999; Russell et al., 2011; Reuter, 2001). As of year 2000, 32 species of *Bifidobacterium* had been identified and all are rod-like, pleomorphic, Gram-positive, non-motile, non-sporulating, non-gas-producing, anaerobic, catalase-negative (except *Bifidobacterium indicum* and *Bifidobacterium asteroides*), saccharolytic microorganisms that exhibit approximately 93% homology in their 16S rDNA sequences (Ventura et al., 2004). They also produce lactic acid and acetic acid, and express fructose-6-phosphate phosphoketolase, which metabolizes hexose phosphate to erythrose-4 phosphate and acetyl phosphate.

Bifidobacteria are invariably the predominant microbes in the gastrointestinal tract soon after birth, representing more than 60% of the total microbiota (Harmsen et al., 2000). Their numbers then wane with time to only a few percent (Franks et al., 1998; Sghir et al., 2000; Langendijk et al., 1995; Claesson et al., 2011). Importantly, the composition of the microbiota in the gastrointestinal tract and in the feces is affected by diet, health, geographical location, and age (Morelli, 2008; Benno et al., 1984; Lay et al., 2005). At the species level, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *B. breve*, and *B. longum* appear to predominate in infants, whereas the number of *Bifidobacterium* species in adults has been shown to be more diverse (Turroni et al., 2012; Matsuki et al., 2004; Takada et al., 2004; Mättö et al., 2004).

B. SAFETY EVALUATION OF *B. breve* M-16V INGESTION

Comprehensive safety testing of probiotics has become the industry standard and current safety analyses include 1) genetic homology analyses that identify genes known to confer toxicity and pathogenicity, 2) functional analyses to evaluate the presence of plasmids, genetic instability, potentially deleterious or undesirable metabolic activities, and 3) *in vivo* analyses to evaluate the potential for pathogenicity or toxicogenicity (Joint FAO/WHO Working Group, 2002; Sanders et al., 2010; Wassenaar and Klein, 2008; Figueroa-Gonzalez et al., 2011). These endpoints and others have been examined for *B. breve* M-16V and the findings are in the following sections.

1. Functional and Genomic Analyses

a. Determination of Antibiotic Resistance

The absence of antibiotic resistance genes in *B. breve* M-16V was determined by searching for genomic sequences with homology to known antibiotic resistance genes found in other strains of bifidobacteria and lactobacilli (Appendix 6). BLASTN analyses showed that the *B. breve* M-16V genome contains regions of homology with Expect-values greater than 0.075 indicating that the *B. breve* M-16V genome does not contain regions homology to sequences in known antibiotic resistance genes.

Functional analyses were also performed by Morinaga Milk Industry, Danone Research, the University of Ghent, and PROSAFE (Table 10). Morinaga compared the sensitivity of *B. breve* M-16V and the type strain *B. breve* ATCC 15700 to antibiotics and found that the minimum inhibitory concentrations (MICs) of the two strains were similar (a difference of ≤ 2 -fold) (Xiao et al., 2010). Danone Research found similar MICs using methods described by Klare et al. (2005), which were confirmed by the University of Ghent (Appendix 7A, B, and C; *B. breve* M-16V referred to Original no. 200). Importantly, all MICs were equal to or less than the cut-off values proposed by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in 2008 (Panel on Additives and Products or Substances used in Animal Feed, 2008). PROSAFE found that *B. breve* M-16V MICs were equal to, if not below, the cut-off values established by PROSAFE for *B. breve* (Appendix 2A and B (*B. breve* M-16V referred to EU-PS38)).

Table 10. Antibiotic Susceptibility Testing of *Bifidobacterium breve* M-16V

Antibiotic	Minimum Inhibitory Concentration (MIC: µg/ml)							
	Facility	Morinaga		Danone Research	U of Ghem	PROGATE		
	FEEDAP cut-off ^a	<i>B. breve</i> ATCC 15700	<i>B. breve</i> M-16V	<i>B. breve</i> M-16V	<i>B. breve</i> M-16V	Cut off ^b	Submitted by Morinaga ^c	Submitted by Danone Research ^d
	<i>B. breve</i>	<i>B. breve</i> ATCC 15700	<i>B. breve</i> M-16V	<i>B. breve</i> M-16V	<i>B. breve</i> M-16V	<i>B. breve</i>	<i>B. breve</i> M-16V	<i>B. breve</i> M-16V
Ampicillin	≤ 2	0.125	0.125	< 1.56	0.25	inconclusive	0.25	0.125
Ampicillin/sulbactam						inconclusive	0.25	0.125
Amoxicillin				< 1.56				
Bacitracin				≤ 3.9				
Chloramphenicol	≤ 4	0.5	1	< 1.56		≤ 2	2	2
Ciprofloxacin		16	8					
Clindamycin	≤ 0.25	≤ 0.032	≤ 0.032	≤ 0.125	0.25	≤ 0.5	≤ 0.032	0.125
Colistin sulphate				125 - 500				
Erythromycin	≤ 0.5	0.125	0.25	≤ 0.125		≤ 0.25	≤ 0.016	0.25
Fusidic acid				15.6		≤ 16	4	8
Gentamicin	≤ 64	64	64	15.6	>32	inconclusive	32	128
Kanamycin		256	512	250 - 500				
Lincomycin				< 1.56				
Linezolid		1	2					
Metronidazol				15.6 - 31.3				
Neomycin		> 256	> 256	62.5				
Oxytetracyclin	≤ 2			< 1.88		≤ 2	1	2
Penicillin G				< 1.52		inconclusive	0.25	0.125
Polymyxin B sulfate				15.6 - 125				
Quinupristin/Dalfopristin	≤ 1			≤ 0.125		≤ 0.25	≤ 0.032	0.125
Rifampicin		≤ 0.125	≤ 0.125					
Streptomycin	≤ 128	128	128	14	96	inconclusive	32	128
Teicoplanin						inconclusive	≤ 0.125	≤ 0.125

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Table 10. Antibiotic Susceptibility Testing of *Bifidobacterium breve* M-16V

Antibiotic	Minimum Inhibitory Concentration (MIC; µg/ml)							
	Facility	Morinaga		Danone Research	U of Ghent	PROSAFE		
	FEEDAP cut-offs				Submitted by Danone Research ¹	cut-offs	Submitted by Morinaga	Submitted by Danone Research
	<i>B. breve</i>	<i>B. breve</i> ATCC 15700	<i>B. breve</i> M-16V	<i>B. breve</i> M-16V	<i>B. breve</i> M-16V	<i>B. breve</i>	<i>B. breve</i> M-16V	<i>B. breve</i> M-16V
Tetracyclin	≤ 8	0.5	0.5	< 1.50	2			
Trimethoprim		2	4	31.3		inconclusive	16	64
Trimethoprim/ Sulfamethoxazole							32	128
Vancomycin	≤ 2	1	0.5	0.25		≤ 1	0.5	0.5
Virginiamycin		0.031	0.063					

¹Unfilled boxes indicate that the analysis was not performed.

²MIC cut-off values for the species *B. breve* proposed by FEEDAP (Panel on Additives and Products or Substances used in Animal Feed, 2008)

³Adopted from Xiao et al., 2010.

⁴MIC cut off values established by PROSAFE.

⁵Full PROSAFE reports stating MICs from *B. breve* M-16 submitted by Morinaga and Danone Research are provided in Appendix 2A and B, respectively. *B. breve* M-16V is referred to EU-PS39 in Appendix 2B.

⁶The full report from University of Ghent is provided in Appendix 7A, B, and C. Numbers represent average of two MICs.

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b. *Presence of Plasmids*

Plasmids have been found in bifidobacteria, including *B. breve* (Sgorbati et al., 1982; Bourget et al., 1993). To determine whether or not *B. breve* M-16V contains plasmids, the bacteria were lysed, and genomic and extra-genomic material was resolved by agarose gel electrophoresis as described by Anderson and McKay (1983). Compared to *Lactobacillus paracasei*, which often contains one or more plasmids (Desmond et al., 2005; Kojic et al., 2010), no low molecular weight DNA bands were detected in *B. breve* M-16V (Figure 8). Furthermore, genomic sequencing of *B. breve* M-16V found no sequences other than those contained within its single chromosome. Together these results demonstrate that *B. breve* M-16V does not contain plasmids.

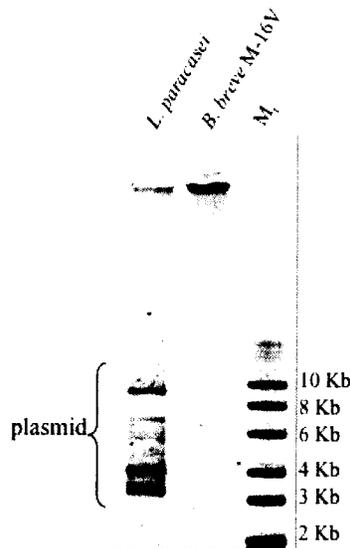


Figure 8. Absence of plasmids in *Bifidobacterium breve* M-16V. Plasmids were extracted from *Lactobacillus paracasei* and *B. breve* M-16V with 1% SDS, precipitated, resuspended, and resolved by agarose gel electrophoresis. M_r denotes the molecular weight marker.

c. *Genomic Analysis for Known Toxins and Pathogenic Markers*

To assess the toxic and pathogenic potential of *B. breve* M-16V, predicted amino acid sequences, and, in some case, mRNA sequences of genes expressed by *B. breve* M-16V were compared to those expressed by toxic and pathogenic bacteria using BLASTP. Although *B. breve* M-16V expressed a variety of potential gene products that are highly homologous (E-values less than 1×10^{-50}) to those gene products expressed by *Arcanobacterium pyogenes*, *Pseudomonas aeruginosa*, *Clostridium perfringens* strain 13, and *Staphylococcus aureus* N315 (Appendix 8A), subsequent BLASTP analyses also showed that these potential gene products are also highly homologous (E-values less than 1×10^{-41}) to gene products expressed by other species of *Bifidobacterium* including *B. infantis*, *B. longum*, *B. catenulatum*, *B. adolescentis*, and *B. bifidum* (Appendix 8B). Importantly, BLASTP analyses only identify regions of homology and do not evaluate the expression and functionality of the homologous regions. Furthermore, the toxicity and/or pathogenicity of bacteria is dependent on the coordination of environmental stimuli and the expression and activation of a variety of other gene products (Wassenaar and Gastra, 2001). Thus, the regions of homology to *A. pyogenes*, *P. aeruginosa*, *C. perfringens* strain 13, and *S. aureus* N315 may not confer toxicity or pathogenicity. Studies evaluating the toxicity and pathogenicity of *B. breve* M-16V are included in section V.B.2 and indicate that *B. breve* M-16V behaves in a fashion similar to *B. longum* BB536, which is GRAS for use in conventional foods (GRN 268).

d. *D-lactic Acid Production*

The production of D- and L-lactic acid isomers by *B. breve* M-16V, *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2772, *Lactobacillus rhamnosus* LW 744, and the type strain *B. breve* ATCC15700 was determined by enzymatic assays involving D- and L-lactate dehydrogenase (Appendix 4). *L. delbrueckii* subsp. *bulgaricus* NCFB2772, *L. rhamnosus* LW 744 are known producers of D-lactic and L-lactic acid, respectively. *B. breve* M-16V produced L-lactic acid but no D-lactic acid (Figure 9A) in a manner similar to *L. rhamnosus* LW 744 and *B. breve* ATCC15700 (Figure 9B).

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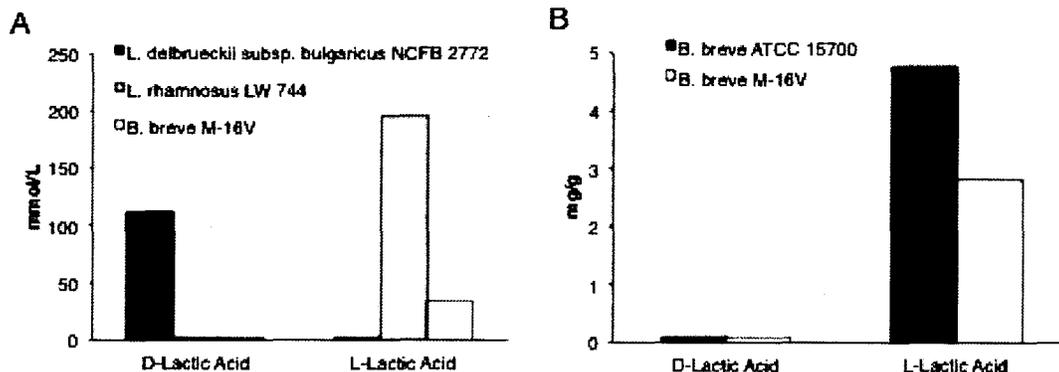


Figure 9. D and L-lactic acid production by *Bifidobacterium breve* M-16V.

A) D and L-lactic acid production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2772, *Lactobacillus rhamnosus* LW 744, and *B. breve* M-16V cultured in MRS broth.
B) D and L-lactic acid production from *B. breve* ATCC 15700 and *B. breve* M-16V cultures.
D and L lactic acid production was determined culturing the different strains in MRS medium and the quantifying the amount of D and L lactic acid using enzymatic assays involving D- and L-lactate dehydrogenase (Appendix 4).

e. Bile Salt Deconjugation

Bile salt hydrolase activity is common to all bifidobacteria, including *B. breve* (Tanaka et al., 1999). *In vitro* analyses showed that *B. breve* M-16V hydrolyzed the conjugated bile acids taurocholic and glycocholic acid to the primary bile acid cholic acid and hydrolyzed glycochenodeoxycholic and taurochenodeoxycholic acid to chenodeoxycholic acid (Table 11). The analyses also showed the *B. breve* M-16V did not dehydroxylate cholic acid and chenodeoxycholic acid to the secondary bile acids deoxycholic and lithocholic acids (Table 11). The methods used to determine *B. breve* M-16V bile salt deconjugation and dehydroxylation are described in Appendix 4.

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Table 8. Bile Salt Deconjugation Activity of <i>Bifidobacterium breve</i> M-16V ¹		
Reaction	Output	
Substrate (Bile acid) → Product	Remaining amount of substrate (μM)	Amount of product (μM)
taurocholic acid → cholic acid	UQ ²	45.3 ± 5.9
glycocholic acid → cholic acid	5.3 ± 6.1	58.9 ± 24.2
taurochenodeoxycholic acid → chenodeoxycholic acid	59.8 ± 11.3	25.5 ± 11.3
glycochenodeoxycholic acid → chenodeoxycholic acid	0 ³	47.7 ± 21.7
cholic acid → deoxycholic acid ⁴ and lithocholic acid ⁴	40.6 ± 23.0	ND ⁵
chenodeoxycholic acid → deoxycholic acid ⁵ and lithocholic acid ⁵	22.6 ± 7.8	ND

¹Bile salt deconjugation was determined by culturing *B. breve* M-16V 0.1 mM bile acid for 16 hr, extracting the bile acids and deconjugated products using nordeoxycholic acid, hydrochloric acid and ethyl acetate, and quantifying the bile acid and deconjugated product by HPLC (Appendix 4).
²UQ denotes “unquantifiable” because no method was available to quantify the amount of Taurocholic acid.
³Glycochenodeoxycholic acid was not detected in the medium after culturing and therefore, its concentration was assumed to be below the limit of detection.
⁴Deoxycholic acid and lithocholic acid are secondary bile acids.
⁵ND denotes not detected.

f. *Biogenic Amines*

Biogenic amines are low molecular weight organic bases formed in foods by the microbial decarboxylation of amino acids or by the transamination of aldehydes and ketones by amino acid transaminases. The ingestion of high amounts of biogenic amines can induce facial flushing, sweating, rash, a burning taste in the mouth, diarrhea, cramps, respiratory distress, swelling of the throat, and blurred vision (reviewed in Ladero et al., 2010). To determine whether the genome of *B. breve* M-16V contains genes capable of conferring amino acid decarboxylase activity, potential open reading frames (ORFs) were identified and compared to ORFs in the NCBI genomic database by BLASTP as described by Kosuge et al. (2006). No regions of homology were found, indicating that *B. breve* M-16V does not express genes known to induce biogenic amine formation.

Functional analyses evaluating the formation of biogenic amines was indirectly determined *in vitro* by analyzing the pH of a solution containing amino acid substrates before and after the addition of the *B. breve* M-16V as described in Appendix 4. Compared to *Lactobacillus buchneri* L586 and *Enterococcus faecalis* E213, which form histamine and

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tyramine, respectively, there was no appreciable increase in pH when *B. breve* M-16V was added to histidine- and tyrosine-containing solutions (Figure 10). These results indicate that *B. breve* M-16V does not form biogenic amines.

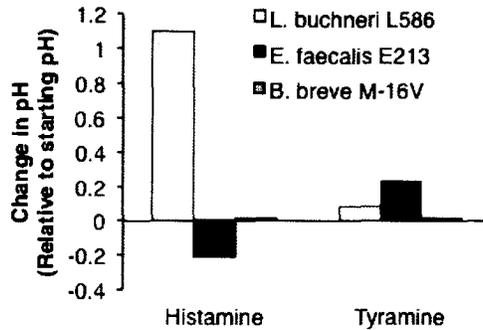


Figure 10. Biogenic amine production by *Lactobacillus buchneri* L586, *Enterococcus faecalis* E213, and *Bifidobacterium breve* M-16V.

Biogenic amine production was determined by culturing the bacteria in potassium phosphate buffer with or without histidine or tyrosine and measuring the change in pH after five hr (Appendix 4).

g. Ammonia Production

Ammonia production by *B. breve* M-16V was determined *in vitro* using a colorimetric assay as described in Appendix 4. Compared to medium harvested from *E. faecium* ATCC 19434 and *L. rhamnosus* LW744, two types of bacteria known to produce and not produce ammonia, respectively, ammonia was undetectable in the medium harvested from *B. breve* M-16V cultures (Figure 11).

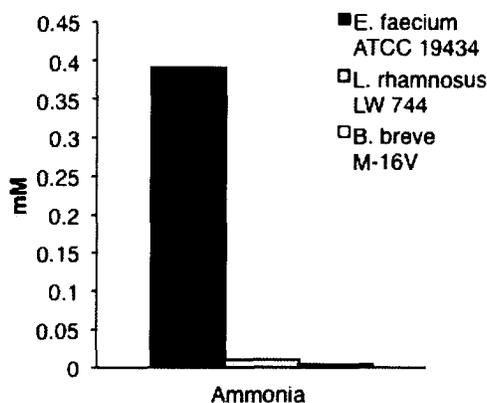


Figure 11. Ammonia production by *Enterococcus faecalis* ATCC 19434, *Lactobacillus rhamnosus* LW744, and *Bifidobacterium breve* M-16V.

Ammonia production was determined as described by Di Giorgio (1974) and Lin and Visek (1991) (Appendix 4).

h. Azoreductase and Nitroreductase Activity

Previous studies have shown that *B. breve* does not have azoreductase or nitroreductase activity (Nakamura et al., 2002). To determine whether the genome of *B. breve* M-16V contains genes capable of conferring azoreductase and nitroreductase activity, potential ORFs were identified and compared to sequences in the NCBI genomic database (nr, 20120604) using BLASTP as described by Kosuge et al. (2006). *B. breve* M-16V contained no regions with homology to other genes known to confer azoreductase activity. However, three potential ORFs had significant homology (E-values less than 1×10^{-50}) to nitroreductases expressed by other types of bifidobacteria and two of the three potential ORFs, g1083 and g1373, had less than 50% homology to nitroreductase genes expressed by other types of bacteria (Appendix 9).

To confirm that *B. breve* M-16V did not spontaneously acquire azoreductase activity, the bacterium was grown on agar plates containing Direct Blue 15, Sunset Yellow FCF, and Amaranth food dye E213, and the plates were analyzed for discoloration as described in Appendix 4. Compared to *C. perfringens* ATCC 13124 and *Lactobacillus fermentum* L421, which reduce and do not reduce azo dyes, respectively. No discoloration was observed for *B. breve* M-16V (Table 12).

Strain	Amaranth	Direct Blue	Sunset Yellow
<i>Clostridium perfringens</i> ATCC 13124	+	+	+
<i>Lactobacillus fermentum</i> L421	-	-	-
<i>B. breve</i> M-16V	-	-	-

¹Azoreductase activity was determined by growing the bacteria on brain heart infusion agar plates containing Direct Blue 15, Sunset Yellow FCF, and Amaranth food dye E213, and analyzing the plates for discoloration (Appendix 4).

Nitroreductase activity was functionally evaluated by incubating culture medium harvested from a *B. breve* M-16V culture with para-aminobenzoic acid (PABA), and quantifying the reduction of PABA using a colorimetric assay as described in Appendix 4. No nitroreductase activity was found in the *B. breve* M-16V culture medium, despite the presence of genes with less than 50% homology to nitroreductase genes expressed by other types of bacteria. Therefore, *B. breve* M-16V does not produce any substances that have nitroreductase activity.

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i. Hemolytic Potential

The hemolytic potential of *B. breve* M-16V was assessed by identifying potential ORFs and compared to the NCBI genomic database (nr, 20120604) as described by Kosuge et al. (2006). BLASTP analyses revealed that the genome of *B. breve* M-16V contains one gene, g0647, having homology to other bifidobacterial gene products that may be involved in hemolysis (Appendix 10).

Hemolytic activity was evaluated by plating and culturing *B. breve* M-16V on agar plates containing sheep blood for 72 hr as described in Appendix 4. In contrast to *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, which is known to cause red blood cell lysis, hemolysis was undetectable for *B. breve* M-16V and *B. longum* subsp. *longum* BB536, a strain of bifidobacteria that has already been GRAS (GRN 268) (Figure 12). A study conducted by Sanquin Research also found that *B. breve* M-16V had no deleterious effects on erythrocytes (Appendix 11, *B. breve* M-16V referred to B602).

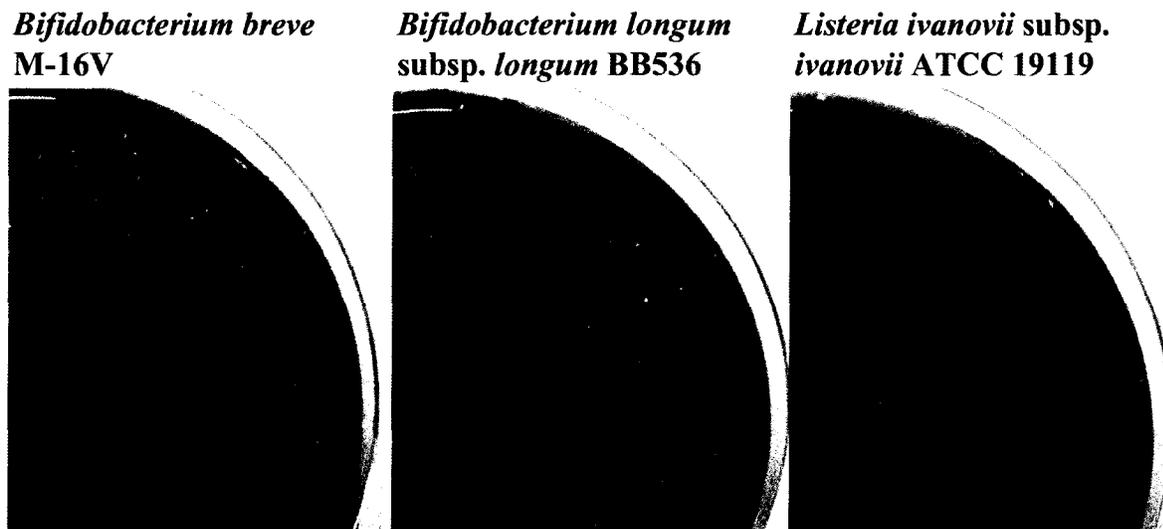


Figure 12. Hemolytic potential of *Bifidobacterium breve* M-16V. *Bifidobacterium longum* subsp. *longum* BB536, and *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 were cultured on GAM agar containing sheep blood. Zone hemolysis was determined 24 hr later (Appendix 4).

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j. Platelet aggregation

The potential for *B. breve* M-16V to induce platelet aggregation and cell death, as measured by cell surface expression of phosphatidylserine was evaluated by Sanquin Research (Appendix 12; *B. breve* M-16V is referred to as *B. breve* B602). *B. breve* M-16V did not induce platelet aggregation nor it it induce cell death. Furthermore, BLASTP analyses showed that the *B. breve* M-16V genome does not contain any regions with homology to genes expressed by other bacteria known to cause platelet aggregation.

k. Mucolytic Activity

The mucin layer of the gastrointestinal tract helps protect underlying epithelial cells from digestive enzymes present in gastric juice, shear generated by digestive processes, and ingested pathogens (Johansson et al., 2011). Thus, any alteration in the mucus layer may compromise the host. *B. breve* M-16V was unable to grow in medium or on agar containing mucin and does not induce mucin degradation *in vitro* (Abe et al., 2010).

2. Toxicology and Safety Studies

a. Mutagenicity

The mutagenic activity of *B. breve* M-16V was determined by the induction of reverse mutations in *Salmonella typhimurium* strains TA98 and TA100 (Ames Test; preincubation method) in the presence or absence of a liver S9 metabolic activation system as described in Appendix 4 (Table 13). Positive control substances furylfuramide (AF-2) and 2-aminoanthracene (2-AA) produced highly elevated numbers of mutant colonies confirming the sensitivity of the test bacteria to known mutagens. Concentrations of 0, 40, 200, 1000 and 5000 µg of *B. breve* M-16V did not produce a positive mutagenic effect in the *Salmonella* test strains, as judged by the absence of a minimum 2-fold increase in reverse mutants in the tests with and without S9. *B. breve* M-16V was, therefore, concluded to be non-mutagenic in this test system.

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Table 13: Absence of Mutagenicity of *Bifidobacterium breve* M-16V¹

Sample	Concentration (µg/plate)	S-9 mg	Number of revertant colonies/plate					
			<i>Salmonella typhimurium</i> TA98			<i>Salmonella typhimurium</i> TA100		
			1 st	2 nd	Mean	1 st	2 nd	Mean
M-16V	5,000	-	51	45	48	104	121	113
	1,000	-	58	65	61	135	120	128
	200	-	42	55	49	142	149	146
	40	-	54	51	53	142	114	128
	0	-	50	58	54	142	164	153
M-16V	5,000	+	51	42	47	92	103	98
	1,000	+	55	55	55	96	100	98
	200	+	57	59	58	96	100	98
	40	+	56	41	49	92	114	103
	0	+	53	59	56	92	107	100
AF-2 ¹	0.1	-	506	492	499	---	---	---
	0.01	-	---	---	---	777	736	757
2-AA ²	0.5	+	506	492	499	---	---	---
	1.0	+	---	---	---	600	630	615

¹Mutagenicity was determined by incubating M-16V powder, furoylfuranamide (AF-2), or 2-aminoanthracene (2-AA) in agar medium containing *Salmonella typhimurium* TA100 or TA98 with or without the metabolic activation agent S9, and counting the colonies afterwards (Appendix 4).

b. Single Oral Dose Toxicity

The safety of *B. breve* M-16V was also evaluated in a single dose rat study; a powder containing 2.3×10^{11} cfu/g was suspended in saline and administered by gavage to 2 groups of 10 male and 10 female three week-old Crj:CD (SD) rats at 3,000 mg/kg (6.9×10^{11} cfu/kg body weight) or 6,000 mg/kg (1.4×10^{12} cfu/kg body weight) (Abe et al., 2009b). A control group of 10 male and 10 female rats was administered a corn starch suspension. After administration, general signs (external appearance, nutritional condition, posture, behavior, and abnormalities in the excreta) were monitored and body weights were measured continuously for a test period of 14 days. Animals that survived at the end of the observation period were terminated by exsanguination and subjected to necropsy.

Observations of general signs revealed no deaths or abnormalities related to treatment. For males, body weight was significantly lower in the 6,000 mg/kg group than in the 3,000 mg/kg group and the control group on days 8 and 10. However, the differences were not evident by days 12 to 14. For females, the changes in body weight in both high and low dose groups

were equivalent to those in the control group. All the animals were necropsied at the end of the observation period. All the organs (brain, pituitary thyroid gland (including the parathyroid gland) adrenal gland, thymus, spleen, heart, lung (including the bronchial tree), salivary glands, (including the submandibular gland and sublingual gland) liver, kidney, testis, prostate, seminal vesicle, ovary, and uterus) examined had no abnormal gross or histopathological findings attributable to treatment.

c. 13-Week Repeated Oral Toxicity

A 90-day repeated dose oral toxicity study using 5-week old Crj:CD (SD)IGS rats was conducted in compliance with Japanese guidelines (Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives (1996) and Guidelines for Repeated Dose Toxicity Test (1999) by the Ministry of Health and Welfare, Japan) (Abe et al., 2009b). Rats were randomized into one test and one control group of 10 male and 10 female rats/group. *B. breve* M-16V powder containing 2.3×10^{11} cfu/g suspended in saline was administered by gavage at a dose of 1,000 mg/kg/day (i.e. 2.3×10^{11} cfu/kg/day). Rats in the control group were administered cornstarch suspension by gavage.

All animals were observed for clinical signs three times a day. Body weight and feed consumption were measured approximately every three days over the course of the study. Ophthalmological examinations were conducted before and at the end of the study. Urinalysis was performed using urine collected at week 13 (day 85 to day 87). Four-hour urine was collected under fasting conditions but with free access to water. Then 20-hr urine was collected with free access to feed and water. The 4-hr urine was analyzed for pH, protein, ketones, glucose, occult blood, bilirubin, urobilinogen, color, and sediment. Urine volume was calculated by totaling the amount of the two urine samples. For electrolytes, the amount excreted per day was calculated.

At the end of the test period, blood was collected for the following hematologic measurements: red blood cell count (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte percentage, platelet count, white blood cell count (WBC), differential white blood cell count, prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen measurements. At the same time as blood collection for hematological examination, blood was also collected and serum was separated for clinical chemistry analyses: alkaline phosphatase (ALP), total cholesterol (TC), triglycerides (TG), phospholipids (PL), total bilirubin (T-bil), glucose, blood urea nitrogen (BUN), creatinine,

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sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (P), total protein (TP), albumin, and the albumin/globulin (A/G) ratio. Plasma obtained from the same blood was analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and γ -glutamyl transpeptidase (γ -GTP). At termination, animals were subjected to necropsy, organ weight measurements and gross and histopathological examination. The histopathological examinations were conducted on: cerebellum, spinal cord (cervical, thoracic, lumbar), sciatic nerve, thoracic aorta, heart, trachea, lung (including bronchial tubes), tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, salivary glands (submandibular and sublingual), liver, pancreas, pituitary, thyroid gland (including parathyroid gland), adrenal, thymus, spleen, mesenteric lymph node, mandibular lymph node, kidneys, urinary bladder, testes, epididymis, prostate, seminal vesicle, uterus, ovaries, vagina, mammary gland, oviduct, skin (inguinal region), eye, optic nerve, Harderian gland, sternum (including bone marrow), femur (including bone marrow), femoral skeletal muscle, nasal cavity, Zymbal gland, and any lesion detected macroscopically.

No abnormal clinical signs attributed to test material were noted during the study period. No significant differences in body weights in the rats administered *B. breve* M-16V were seen compared to the control group for either males and females. Feed consumption in the test group was not different compared to the control group for either males and females. No abnormalities were seen on ophthalmological examination and there were no significant differences in urinalysis parameters between test and control groups for both males and females. For hematology, there were no significant differences related to the administration of *B. breve* M-16V for any measured parameter in both males and females. A statistically significantly higher MCH value in males administered *B. breve* M-16V was noted compared to the control (19.5 ± 0.4 vs. 18.8 ± 0.5 pg); however, there was no difference in this parameter in females. In clinical chemistry, there were no significant changes related to the administration of M-16V in both males and females compared to controls, although the total bilirubin of male (but not female) rats administered *B. breve* M-16V was significantly different from the control (0.1 ± 0.0 vs. 0.0 ± 0.1 mg/dl). No significant differences were seen between treated rats and controls for absolute organ weights. No abnormalities related to treatment were seen in the gross or histopathological examinations.

In conclusion, there were no adverse treatment related effects seen from administration of M-16V to rats at a level of 2.3×10^{11} cfu/kg/day for 90 days. An acceptable level of intake of 1.38×10^{11} cfu/day for a 60-kg individual may be derived by utilizing a typical 100-fold safety factor applied to the test level of 2.3×10^{11} cfu/kg/day.

d. Tissue Translocation Potential

B. breve is one of a variety of bacteria found in the human gastrointestinal tract (Matsuki et al., 1999; Haarman and Knol, 2005). In healthy humans, the gut microbiota are contained inside the gastrointestinal tract, but in some circumstances they translocate (penetrate) into the surrounding tissues and produce pathological effects. To determine whether *B. breve* M-16V was capable of translocating the epithelial lining of the gastrointestinal tract, mice were fed *B. breve* M-16V for five days, treated with the immunosuppressive agent cyclophosphamide 4, 6, and 8 days after beginning bacterial treatment, and euthanized four days later (Appendix 4). Blood and liver, spleen, and mesenteric lymph node homogenates were cultured on BL agar under anaerobic conditions and the resulting colonies were counted. *B. breve* M-16V and *B. longum* subsp. *longum* BB536 were not detected in the blood, liver, spleen, and mesenteric lymph nodes. These results indicate that *B. breve* M-16V did not translocate outside the gastrointestinal tract, even in immunocompromised hosts.

e. Intravenous Challenge

Bacterial-induced toxicity was determined by intravenously administering increasing amounts of *B. breve* M-16V or *B. longum* BB536 to healthy and immunocompromised mice and monitoring the mortality rate over 14 days (Appendix 4). Toxicity of the opportunistic human pathogen *P. aeruginosa* was used as a positive control and induced death in healthy mice at approximately 10^6 cfu (Figure 13A). In contrast, *B. breve* M-16V caused deaths in a dose-dependent fashion at doses greater than 10^9 cfu, which was similar to that induced by *B. longum* BB536 (Figure 13A). In immunocompromised mice, *B. breve* M-16V caused deaths at doses greater than 0.3×10^9 cfu, which was similar to what was observed in healthy mice (Figure 13B, compare *B. breve* M-16V in left and right panels), and was approximately 10-fold less than that induced by *B. longum* BB536 (Figure 13B). These results show that *B. breve* M-16V is not highly toxic or pathogenic.

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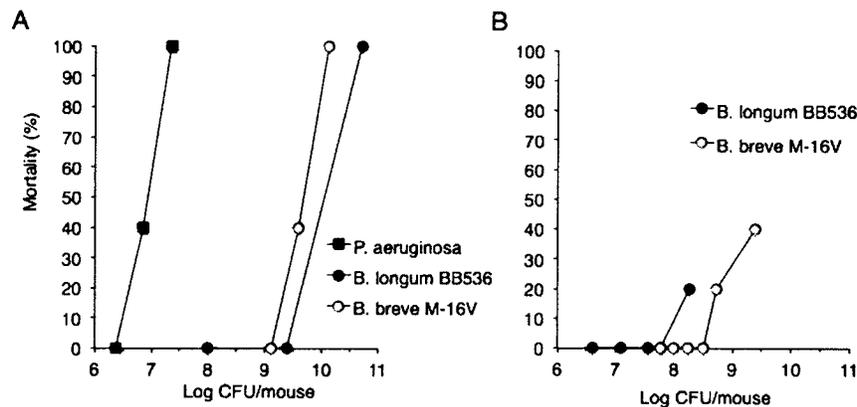


Figure 13. Intravenous effects of *Bifidobacterium breve* M-16V.

(A) *Pseudomonas aeruginosa*, *B. breve* M-16V, or *Bifidobacterium longum* BB536 was injected intravenously into healthy mice and lethality was monitored over 14 days. (B) *B. breve* M-16V or *B. longum* BB536 was injected intravenously into immunocompromised mice and lethality was monitored over 14 days (Appendix 4).

3. Clinical Studies

Numerous clinical studies have administered *B. breve* M-16V and other strains of bifidobacteria to infants, children, and adults. In general, all strains of bifidobacteria are well-tolerated and safe. No studies have reported adverse effects associated with the consumption of *B. breve* M-16V. Only three case reports of opportunistic infections associated with bifidobacteria were found during an extensive literature search (Hata et al., 1998; Nakazawa et al., 1996; Ohishi et al., 2010).

a. Studies in Infants

i. Studies of *B. breve* M-16V

Morinaga's *B. breve* M-16V has been tested in 12 studies (15 publications) in health-compromised and/or premature infants (Table 14) (Bennet et al., 1992; Akiyama et al., 1994; Yamada et al., 2002; Hattori et al., 2003; Sato et al., 2003; Ishizeki et al., 2004; Li et al., 2004; Taniuchi et al., 2005; Fujii et al., 2006; Wang et al., 2007; Umeda et al., 2010; van der Aa et al., 2010; van der Aa et al., 2011; van der Aa et al., 2012; De Kivit et al., 2012). Three studies evaluated the effects consuming 1.0×10^{10} cfu *B. breve* M-16V/d or greater in a total of 65 infants from 1 to 3 mo (Taniuchi, et al. 2005; Hattori et al., 2003; Van der Aa, et al. 2010, 2011, and 2012; De Kivit et al., 2012), four studies evaluated the effects of consuming 9.0×10^9 cfu *B. breve* M-16V/d to 1.0×10^9 cfu *B. breve* M-16V/d in a total of 222 infants from 5 d to 9 mo

(Bennet et al., 1992; Fujii et al., 2006; Sato et al., 2003; Yamada et al., 2002), and 5 studies evaluated the effects of less than 1.0×10^9 cfu *B. breve* M-16V/d in a total of 110 infants from 16 d to 8 wk (Akiyama et al., 1994; Ishizeki et al., 2004; Umeda et al., 2010; Wang et al., 2007; Li et al., 2004). Weight gain was not compromised in infants receiving *B. breve* M-16V, although measurement of growth was not necessarily a primary endpoint in many of the studies reported in the literature. According to a report on clinical testing of infant formulas with respect to nutritional suitability for term infants (American Academy of Pediatrics, 1988), such studies require a minimum of 28 subjects of a specified sex in each of two groups in order to detect a 3 g/d difference in weight gain ($p < 0.05$; power of 0.8 in a one-tailed test).

Taniuchi and co-workers (2005) enrolled 17 infants aged 3.1-18.5 mo having cow's milk hypersensitivity and atopic dermatitis plus $< 30\%$ *Bifidobacterium* spp. in their intestinal microbiota to receive either *B. breve* M-16V at 5 or 15×10^9 cfu/d (oral) ($n = 10$) or no intervention ($n = 7$) for 3 mo after receiving a hydrolyzed casein formula during a ≥ 2 wk run-in period. Fecal microbiota analyses were carried out and allergic symptom scores were assessed at 1, 2, and 3 mo. The treatment group experienced an increase in the proportion of fecal *Bifidobacterium* spp. during the study while the control group did not. Cutaneous symptom scores were significantly decreased in the treatment group at 1, 2, and 3 mo ($p = 0.0255$, 0.0147 and 0.0378, respectively), versus baseline levels. However, cutaneous symptom scores were also reduced at 1 and 3 mo in the control group ($p = 0.0394$ and 0.0407, respectively), versus initial scores. The total allergic score was significantly reduced at all time points in the treatment group, versus baseline levels ($p = 0.0156$, 0.0142, and 0.0359, respectively), while no reduction was observed in the control group. The authors made no mention of adverse effects.

Hattori and co-workers (2003) enrolled 15 infants (8.8 ± 4.3 mo old) having atopic dermatitis, intestinal microbiota deficient in *Bifidobacterium* species, and who had received extensively hydrolyzed casein milk for at least 2 wk. The researchers administered a daily dose of 0.5×10^{10} or 1.5×10^{10} cfu/d to eight infants for 1 mo. Seven infants served as controls. The hydrolyzed casein formula also contained 0.13 g raffinose/100 mL milk. Both the cutaneous symptom score and the total allergic score were significantly reduced in the treatment group versus the control group ($p=0.0176$ and 0.0117, respectively). Fecal samples contained more *Bifidobacterium* species and a higher proportion of anaerobic bacteria versus total aerobic bacteria in the treatment group, versus baseline levels ($p<0.05$ for all). The authors made no mention of adverse effects.

Van der Aa and co-workers carried out a study in infants with atopic dermatitis that were exclusively formula-fed (Van der Aa 2010, 2011, and 2012; De Kivit et al., 2012). Ninety infants

ages less than 1 mo to 7 mo old having atopic eczema dermatitis syndrome were randomized to receive either control formula ($n = 44$) or the supplemented formula ($n = 46$) for 12 wk. It was noted that infants ≤ 6 mo old received starting formula while those > 6 mo received follow-on formula. Infants were assigned to receive an extensively hydrolyzed cow's milk-based formula either without ($n=44$) or with ($n=46$) 1.3×10^9 cfu *B. breve* M-16V /100 mL formula and 0.8 g of 9:1 short chain galactooligosaccharides (GOS):long chain fructooligosaccharides (FOS) per 100 mL formula for 12 wk. Assuming the infants consumed approximately 800 ml of formula per day, this concentration of *B. breve* M-16V results in an intake of 1.04×10^{10} cfu/day. The primary endpoint was SCORAD (SCORing Atopic Dermatitis) values during the study, versus baseline; secondary parameters included the SCORAD extent, intensity and subjective scores, parental quality of life, respiratory symptoms, steroid use, gastrointestinal symptoms, eosinophilic granulocytes in serum, IgE concentration, IgG4 concentration, ratio of IgE/IgG4, IL-5, IgG1, cutaneous T cell-attracting chemokine (CTACK), thymus and activation regulated cytokine (TARC, also known as ABCD-2 and CCL-17), T-cell phenotypes, and fecal parameters. Safety parameters measured included feces frequency and consistency, adverse events, drop-outs, growth, blood safety parameters (eosinophilic granulocyte count, liver and renal function, cutaneous T cell attracting chemokine, thymus and activation-regulated chemokine, total IgG1, IgG4, IgE levels and milk-, peanut-, egg-, fish-, cat-, and dust mite-specific IgE levels) and compliance. The study was statistically powered at 5% significance and 80% power to detect a 25% reduction in SCORAD between the groups. The intent-to-treat population encompassed 89 infants (44 control/45 treatment), while the per-protocol population consisted of 78 infants (41 control/37 treatment). Seven infants terminated the study early (2 in the control group/5 in the treatment group). Reasons for dropping out included: serious adverse event relating to cow's milk allergy (1 treatment), personal reasons (2 treatment), infant did not accept study formula (1 control/1 treatment), protocol violations (1 control); use of antihistamines (1 control); stopped intake of formula after 1 mo (1 treatment). More infants in the control group experienced constipation vs. the treatment group ($p = 0.012$). There was no significant difference in the occurrence of respiratory symptoms, other gastrointestinal symptoms besides constipation, other infections or other symptoms between the two groups. Blood parameters (alanine transaminase, ALT; aspartate transaminase, AST; urea; creatinine, albumin) and growth parameters (z-score for weight; z-score for length; head circumference) did not differ between the groups. There were no differences between control and treatment groups regarding serious adverse events besides constipation. Antibiotic use was higher in the control group vs. treatment group (not statistically significant). Each published report by Van der Aa and co-workers focused on different endpoints that were measured during the trial.

The Van der Aa (2010) report discussed the effect of *B. breve* M-16V and 9:1 scGOS:lcFOS on the severity of atopic dermatitis in infants. In this study, in a specific subpopulation of the study population (IgE+), the severity atopic dermatitis was significantly decreased in the synbiotic group ($n=24$) versus the placebo group ($n=24$) ($P=0.04$) at 12 wk. For the entire study population, bifidobacteria, expressed as a percent of the total fecal biota, were significantly increased in the synbiotic group vs. control at wk 1 ($P=0.045$) and wk 12 ($P<0.001$). At 12 wk, the proportion of fecal biota accounted for by *Clostridium lituseburens*/*C. histolyticum* and *Eubacterium rectal*/*Clostridium coccooides* was decreased in the synbiotic group vs. the control group ($P= 0.02$ and <0.001 , respectively). At study's end, the fecal butyric, isobutyric and isovaleric acid decreased in the synbiotic group vs. placebo ($P=0.04, 0.02, 0.02$), while the fecal pH, L-lactate, D-lactate and acetic acid were significantly increased in the synbiotic group vs. control ($P=0.001, <0.001, <0.001$), suggesting exposure to bifidobacteria had occurred. Fecal consistency, dry stool episodes, constipation, and diaper dermatitis were also reduced in the treatment group vs. control ($P=0.05, 0.001, 0.01, \text{ and } 0.008$). Growth, weight, renal and liver function did not differ between the two groups. Diarrhea and gastroenteritis occurred equally in both groups. Adverse event incidence was similar between the two groups. There were no serious events in the control group and two serious adverse events in the synbiotic group: hospitalization due to respiratory syncytial virus bronchiolitis and severe cow's milk allergy. The authors did not consider any of these adverse events to be treatment-related. The synbiotic formula was reported to be well tolerated.

The Van der Aa report published in 2011 mentions that, at 1 yr after the 12 week exposure (52 wk after study baseline) to an extensively hydrolyzed cow's milk formula either with or without the added synbiotics mentioned above, the prevalence of asthma-like symptoms was decreased in the treatment group ($n=36$) versus control ($n=39$) for the specific endpoints of frequent wheezing ($p=0.04$), wheezing apart from colds ($p=0.056$), wheezing and/or noisy breathing apart from colds ($p=0.001$), asthma medication use ($p=0.049$) and reduced new use of asthma medication at follow-up vs. baseline ($p=0.02$).

The Van der Aa report published in 2012 mentions that the authors did not find a beneficial effect of the synbiotic formula described above on the severity of infant atopic dermatitis, eosinophilic granulocyte count or serum IgE levels at 12 wk. There were no clinically meaningful observations of changes in cytokine levels between the groups, although a few statistically significant *in vitro* assay results were noted.

Bennet and co-workers (1992) orally administered 3×10^9 cfu *B. breve* BB-576 (strain M-16V, according to Morinaga) ($n = 3$), or 3×10^9 cfu *B. longum* BB-536 ($n = 3$), or 3×10^9 cfu

Lactobacillus acidophilus LAC-343 ($n = 3$), or a combination of all three strains at the same dose ($n = 2$), or no intervention ($n = 3$) three times per day (total dose of *B. breve* BB-576 = 9×10^9 cfu/d) for 5 d to full-term infants ages less than one mo old to 8 mo-old. Bacteria were administered on the first day after stopping antibiotic treatment. Fecal specimens were taken on the last day of treatment and 5 and 15 d after completing the study. *B. breve* was recovered from 3/3, 2/3, and 0/1 fecal samples taken from the infants receiving only *B. breve* on the last day of bacterial dosing, and 5 and 15 days afterward, respectively. The authors reported that no side effects were noted. This was the only retrieved study that administered M-16V alone and in combination with other bacteria in term infants.

Fujii and co-workers (2006) randomized 19 preterm infants admitted to a neonatal intensive care unit to receive either 1×10^9 cells of *B. breve* M-16V in 5% glucose solution twice daily (nasogastric administration; total of 2×10^9 bacteria per day; $n=11$) or vehicle (5% glucose solution; $n=8$) for 28 d. Blood samples were collected on days 0, 14, and 28 for cytokine measurement. TGF- β levels were elevated in both groups by 14 d versus baseline levels and by 28 d, TGF- β levels were significantly greater in the treatment group versus the control group ($P = 0.005$). There were no differences between the groups for levels of Smad2, Smad4, and Smad7 mRNA. Smad3 mRNA expression was higher in the *B. breve* M-16V group versus control on d 28 ($P = 0.03$). The authors reported that no adverse effects were observed after *B. breve* M-16V supplementation.

Sato and co-workers (2003) enrolled 162 premature infants with a birth weight < 1.5 kg and administered either 1×10^9 cfu *B. breve* M-16V or no intervention until 36 wk old (corrected gestational age). Seventy-five infants received the M-16V strain (28.3 ± 3.1 wk mean gestational age; 1027.3 ± 279.7 g mean birth weight) and 87 infants served as controls (28.6 ± 2.6 wk; 1064 ± 275.0 g). Infants were tube fed. Infection developed in 20/75 infants in the treatment group (26.6%) and in 33/87 infants in the control group (37.9%). Mean duration of hospitalization was 89.6 d in the M-16V group and 102.3 d in the control group. Mean body weights on the calculated date of confinement (estimated delivery date) were 2426.1 g and 2084.0 g in the M-16V and control groups, respectively. The authors made no mention of adverse effects.

The study by Yamada and co-workers (2002) involved 266 infants admitted to a NICU. Infants received either no intervention or 1×10^9 cfu *B. breve* M-16V as a powder dissolved in 2 mL milk on an alternately assigned basis, as they were admitted. *B. breve* M-16V was given for 5 d beginning from the time milk was started. Prior to this study, the methicillin-resistant *Staphylococcus aureus* (MRSA) infection carrier rate was 30.5% of admitted patients and incidence of MRSA infection and neonatal toxic shock syndrome (TSS)-like exanthematous

disease (NTED) was 9.5%. After administration of *B. breve* M-16V the MRSA carrier rate was 12.3% and the incidence of MRSA infection and NTED was 2.2%. However, the detection rate of *P. aeruginosa* increased from 3.9% prior to administration of *B. breve* M-16V to 14.6% afterward. The clinical significance of this observation was not discussed in the translated English language summary that was provided by Morinaga.

Akiyama and co-workers (1994) administered 5×10^8 cells *B. breve biovar a*, otherwise known as *B. breve* M-16V ($n = 5$) or dextrin ($n = 5$) per day intragastrically to very low birthweight premature infants (< 1250 g) until 8 wk of age. All subjects were on artificial ventilation except one infant in the control group. Fecal samples were collected at 1, 2, 4, 6, and 8 wk after birth. The administered strain of *B. breve* was detected in the feces of treated infants at all time points during the study. However, the organism was also detected in fecal samples of the control group. The authors state that the organism was administered without any clinical problem.

Ishizeki and co-workers (2004) studied infants with birth weights ≥ 1000 g and < 2000 g who were admitted to the neonatal intensive care unit (NICU) and were able to begin enteral nutrition within one week of birth. The researchers administered 5×10^8 cfu *B. breve* M-16V/d to 15 infants, a combination of *B. breve* M-16V, *B. infantis* M-63, and *B. longum* BB536 (1.5×10^9 cfu total/d) to 13 infants, and no intervention to the control group of 16 infants. The study was carried out for 6 wk. Rates of detection of bifidobacteria at wk one were 14% (control), 57% (one species), and 100% (three species). By wk 8, two weeks after the end of the intervention, the detection rate of bifidobacteria in the one species group had dropped to 27%. The authors made no mention of adverse effects.

Umeda and co-workers (2010) divided 133 extremely low birth weight infants into a control group ($n=84$; 25.7 ± 2.1 wk; 749.2 ± 164.0 g) and a treatment group ($n=49$; 25.7 ± 1.6 wk; 769.8 ± 165.9 g). The treatment group received 5×10^8 cfu *B. breve* M-16V/d mixed into water for an average of 16 d. Infants participating in the control group were in the study for an average of 20.9 d. Necrotizing enterocolitis (NEC) occurred in 9 infants (10.7%) in the control group and one infant (2.0%) in the treatment group ($p=0.06$). Death by NEC occurred in 5 infants (5.6%) in the control group and no infants in the treatment group. Delayed sepsis (positive blood culture) was observed in 28 infants (33.3%) in the control group and 4 infants (8.2%) in the treatment group ($p<0.01$). In the probiotic group, 23 of 49 infants (46.9%) received fluconazole and zero of 89 infants in the control group received fluconazole. Fungal sepsis occurred in 3 infants in the control group and none in the treatment group. Infants were able to consume 100mL/kg/d of milk on d 20.9 in the control group and on d 16.0 in the treatment

group. The average time needed for infants to return to birth weight was 29.7 and 24.3 d, respectively, for the control and treatment groups ($p < 0.05$)

Wang and co-workers (2007) enrolled 66 premature infants and divided them into three groups according to birth-weight: 1) $< 1000\text{g}$ ($n=22$), 2) $< 1500\text{ g}$ ($n=22$), and 3) $< 2500\text{ g}$ ($n=22$). Within each group, infants received either *B. breve* M-16V (total of 3.2×10^8 cells/d) ($n=11$ per birth-weight group) or no intervention ($n=11$) until discharge, or until 4 wk. Fecal samples were collected at 0, 2, and 4 wk after birth, and breast milk was supplemented with infant formula whenever necessary. By 4 wk after birth, the fecal acetic acid:total short-chain fatty acids (SCFAs) ratio was significantly increased in treatment groups versus controls ($p < 0.05$). Fecal extraction of total SCFAs increased with birth weight and time. The authors state that no adverse effects were observed after supplementation with *B. breve* M-16V. No serious infections or positive blood cultures occurred; nor were C-reactive protein levels elevated.

Li and co-workers (2004) randomized low birth weight infants (1489 g avg wt) to receive either 1.6×10^8 cfu *B. breve* M-16V intragastrically with feeding (suspension in 0.5 mL of 5% glucose in sterile distilled water) twice per day (total dose: 3.2×10^8 cfu) within hours of birth ($n = 10$; mean 7.2 h), the same dosing of *B. breve* initiated within 24 h after birth ($n = 10$; mean 36.5 h), or no supplementation ($n = 10$) for 7 wk. The authors observed increased *Bifidobacterium* spp. counts in fecal samples of the immediate-dosing group by 3.4 ± 2.2 d and by 7.2 ± 3.8 d in the delayed-dosing group ($P < 0.05$). *Enterobacteriaceae* spp. were significantly lower in the immediate-dosing group versus the other two groups at wk 2 of the study ($P < 0.05$), but this difference disappeared after 2 wk. There were no differences in the incidence of NEC, infectious disease or sepsis among the three groups, according to the researchers. The authors also stated that they did not observe any side effects due to the administration of *B. breve*.

ii. Studies of *B. breve*

Four additional studies administering live *B. breve* alone or as part of a mixture with other bacteria have been carried out in infants (Table 14)(Kitajima et al., 1997 (two studies); Kukkonen et al., 2006; Kukkonen et al., 2008 (same study as Kukkonen, et al. 2006 but different endpoints reported); Braga et al., 2011) at doses of 2×10^8 (Kukkonen, et al. 2006 and 2008) to 3.5×10^9 cfu *B. breve*/d (Braga, et al. 2011) for durations ranging from 7 d (Kitajima et al., 1997) up to 6 mo (Kukkonen, et al. 2006 and 2008).

Two studies administered *B. breve* alone (e.g., without the presence of other bacteria). Both studies were carried out by Kitajima and co-workers, and were published by Kitajima et al. in 1997. In the first study, the researchers administered 1×10^9 cfu *B. breve* YIT4010 (BBG) up

to three times per day (total dose = 3×10^9 cfu *B. breve* YIT4010 (BBG)) to sixteen infants (28.3 \pm 2.9 wk gestation; 1052 \pm 328 g) within 7 d of life and for a duration of up to 7 d (median 5 d; range 1-7 d). Another group of 50 infants received the organism after 7 d of life and for at least 7 d (median 14 d; range 7-48 g). In the first study the researchers reported that there were no adverse effects from *B. breve* YIT 4010 itself, aside from two incidents of mild functional ileus which were attributed to the formation of aggregates due to the corn starch present as a filler in the probiotic preparation. In the second study, Kitajima and co-workers (*ibid*) enrolled 97 very low birth weight infants of <1500 g who were randomly allocated to receive $\sim 0.5 \times 10^9$ cells suspended in distilled water ($n = 45$) or distilled water alone ($n = 46$). Two infants died before the study began and three were transferred back to the referring hospital before 3 wk old; another had coarctation of aorta that was diagnosed at 15 d. There was no change in growth patterns of *Staphylococcus*, *Pseudomonas*, and *Candida* in infants receiving intervention. The first dose was given within 24 h of birth, then once a day for 28 d. Meconium and stool samples were collected for anaerobic culture every week for 8 wk. Of the initial treatment group, 91 infants were followed up for 2 mo after birth and 70 were followed up for 3 yr to study their growth. Positive stool culture results were more frequent in the treatment group versus controls across all gestational age groupings (no statistics provided) and number of *B. breve* YIT4010 in the feces increased by two weeks in the treatment group (73% vs. 12% incidence in treatment and control groups by 2 wk). Of 58 study infants whose stool samples had been verified for presence of the bacterium by immunochemical staining, 20 did not actually have the bacterium (17 from control group; 3 from treatment group) whereas 26 did (3 from control group; 23 from treatment group). Infants from whom *B. breve* YIT 4010 was recovered had significantly lower aspirated air volumes on days 13-15 and 23-28 ($P < 0.05$), higher feeding volume intakes from d 10 onward ($P < 0.05$), and increased body weight from weeks 4-8 ($P < 0.05$). Two infants died at 5 and 16 d of age, but it was not stated whether these infants were in the treatment group. The authors state that the trend for better weight gain in the treatment group persisted out to 18 months but was not statistically significant. The authors do not mention the outcome for the infants followed up on for 3 yr.

iii. Studies of *B. breve* in combination with other Probiotics

Two studies have reported on the effects of administering *B. breve* in the context of additional bacterial species or strains (Table 14)(Kukkonen et al., 2006; Kukkonen et al., 2008 (same study as Kukkonen, et al. 2006 but different endpoints reported); Braga et al., 2011). Doses in these studies ranged up to 2×10^8 cfu/d (Kukkonen, et al. 2006 and 2008) and exposure times were up to 6 mo (Kukkonen, et al. 2006 and 2008).

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Kukkonen and co-workers (2006; same study as reported in 2008 but different endpoints) reported on the vaccine response in a sub-population of patients drawn from 1223 infants randomized to an allergy-prevention trial on high-risk infants (safety data from the main study is reported in Kukkonen, et al. 2008). The authors randomized pregnant mothers to receive two capsules/d containing 5×10^9 cfu *L. rhamnosus* GG, 5×10^9 cfu *L. rhamnosus* LC705, 2×10^8 cfu *B. breve* Bbi99, and 2×10^9 cfu *Propionibacterium freudenreichii* ssp. *shermanii* JS ($n = 47$) or placebo (microcrystalline cellulose) ($n = 40$) during the 4 wk prior to delivery. After delivery, infants continued on the same intervention, in the form of the contents of one capsule per day plus 20 drops of sugar syrup containing 0.8 g galacto-oligosaccharides (GOS) (probiotic group only) for 6 mo. Infants were vaccinated against diphtheria, tetanus toxoid, and *Bordetella pertussis* at 3, 4, and 5 mo of age. They also received either Hiberix® or HibTITER® (both for *Haemophilus influenzae* type b, Hib) at 4 mo of age. The authors state that they observed no important side effects during the treatment. Growth and abdominal symptoms were similar between the two groups. Probiotic supplementation in infants at high risk of atopy did not interfere with antibody responses to the study vaccines. Protective (designated as $\geq 1 \mu\text{g/mL}$) levels of anti-Hib IgG antibodies occurred in the probiotic group more frequently versus the placebo group (50 vs. 21%, respectively; $p = 0.020$).

Kukkonen and co-workers (2008) reported the safety-related results from their mother-infant probiotic intervention study. This study enrolled 1223 pregnant mothers carrying infants at high risk for allergy and assigned them to receive two capsules/d of the probiotic mixture detailed above ($n = 506$) or two microcrystalline cellulose placebo capsules ($n = 512$) for the 4 wk prior to delivery. Then the newborns received the contents of one probiotic capsule plus added galacto-oligosaccharides (GOS; 0.8 g) ($n = 468$) or one capsule of microcrystalline cellulose plus no added GOS ($n = 471$) for 6 mo. A 2 yr follow-up evaluation was also carried out on the probiotic- ($n = 461$) and placebo-treated ($n = 464$) infants. Of the initial 1223 randomized mothers, 156 refused to participate, and 49 of their infants were ineligible. Of the ineligible infants, 8 were born prematurely to probiotic-treated mothers and 7 were born prematurely to placebo-treated mothers. At 6 mo, there were 506 infants in the treatment group and 512 in the placebo group. Discontinuation rates for the treatment and placebo groups due to adverse events were as follows: infection (11 vs. 18; treatment vs. placebo), oxygen supplementation (11 vs. 18), vomiting (4 vs. 7) and respiratory infection requiring hospitalization (8 vs. 15). During the 2 yr follow up period, the frequency of respiratory infections was lower in the treatment group vs. the placebo group ($P = 0.023$), and there were fewer total number of respiratory infections in the treatment group vs. the placebo group ($P = 0.009$). Middle ear infections tended to be lower in the treatment group vs. placebo ($P = 0.068$) but gastroenteritis was equally common in both groups. There were no statistically significant differences between

the groups for length, weight or head circumference at 6 and 24 mo. Normal growth was reported for both groups, according to the authors.

Braga and co-workers (2011) randomized 243 preterm infants (750-1499 g) who were admitted to the neonatal intensive care unit to receive either human milk alone ($n = 121$) or human milk supplemented with 3.5×10^7 to 3.5×10^9 cfu of combined *L. casei* plus *B. breve* ($n = 122$), also known as Yakult LB. The intervention started on day 2 of life and continued through day 30 (28 d total). Three deaths occurred in the treatment group and nine deaths occurred in the placebo group prior to beginning the intervention. No cases of NEC (stage ≥ 2 by Bell's criteria) occurred in the treatment group; four cases occurred in the control group. The treatment group experienced a statistically significant decrease in a) the amount of complete transition time of orogastric feeding tube to breastfeeding and in b) the time to reach full enteral feeding in the treatment group vs. control group ($P = 0.03$ and 0.02 , respectively). The authors observed no difference in the occurrence of sepsis or in the number of deaths between the treatment and control groups.

iv. Case Reports

There have only been three reported cases of opportunistic infections associated with *Bifidobacterium* species (Table 14). All of them occurred in health-compromised or vulnerable infants. However, the overall weight of the evidence points to *Bifidobacterium* species as being safe for consumption by infants, children and adults. To-date, there have been three GRAS Notices filed with no questions by the Food and Drug Administration: GRN49 for *B. lactis* strain Bb 12 and *S. thermophilus* Th4; GRN268 for *B. longum* BB536; and GRN377 for *B. animalis* subsp. *lactis* Bf-6.

There have been two case reports of meningitis associated with *Bifidobacterium* species-related infections (Hata et al., 1988; Nakazawa et al., 1996). In both cases the patients had a good clinical outcome. Hata and co-workers reported meningitis in a 37 d old Japanese male infant who was delivered at 37 weeks' gestation (2840 g birth weight) to a mother having Behçet's disease (Hata, et al. 1988), which is an autoimmune disease involving damage to blood vessels. The infant had a slightly distended anterior fontanelle and rectal temperature of 38.6°C . The infant was well-nourished and active, but the white blood cell (WBC) count was elevated and the cerebrospinal fluid contained $74 \text{ WBC}/\text{mm}^3$. The cerebrospinal fluid (CSF) culture grew Gram-positive rods after 24 h, but the peripheral blood culture was sterile. The infant was given *i.v.* cefotaxime for 10 d and the fever abated after 3d, at which time the anterior fontanelle was flat. The fever recurred 12 d after admission to the hospital and the cerebrospinal fluid contained $633 \text{ WBCs}/\text{mm}^3$ and grew the same Gram-positive rods under anaerobic culture. Ampicillin was given *i.v.* for 3 wk and the fever diminished after 2 d on this medication. Cerebrospinal fluid cultures on day 15 were sterile. By day 38, the fever recurred and the cerebrospinal fluid was

positive for the same organism. Ampicillin was given *i.v.* again followed by penicillin G for 26 days. On the 64th day, *i.v.* chloramphenicol was initiated. Cerebrospinal fluid isolates were identified as containing *B. breve*. Anaerobic stool culture from the patient was positive for *B. breve* but the organism was not found in the anaerobic stool and vaginal cultures from the mother. The patient eventually recovered. Repeated cranial computerized tomography scans and craniospinal magnetic resonance imaging were normal prior to discharge. Follow-up examination at eleven months of age indicated normal growth and development and no neurologic abnormalities, according to the authors.

The second case report of neonatal meningitis was published by Nakazawa and co-workers (1996). A male infant delivered via caesarian section at 38 weeks with a birth weight of 2640 g exhibited a fever of 39°C, poor feeding, and a mononuclear cell dominant pleocytosis in the cerebrospinal fluid. On the 4th day after hospital admission, anaerobic Gram-positive rods were detected in a blood culture, and the fever recurred 5 d after discontinuing antibiotics (ampicillin and cefotaxime; duration not stated). Anaerobic culture of the CSF again indicated presence of the same Gram-positive organism. Antibiotic therapy was restarted and gamma-globulin infusions were also given. The patient's serum C-reactive protein levels were negative throughout the episode, indicating low levels of inflammation according to the authors. The bacterium was identified as *B. breve* via gas-liquid chromatography. The route of infection was not identified. After two relapses the patient was cured completely and there were no reported neurological abnormalities.

There was one case report of neonatal sepsis associated with *B. breve* BBG-01 in a female infant diagnosed with omphalocele at 13 wk gestation who was delivered at 37 wk and 2 d of gestation by cesarean delivery (Ohishi et al., 2010). Birth weight was 2060 g with liver and intestine prolapse plus polydactyly of the right hand. Surgery was carried out to correct the omphalocele 4 h after birth and 2 d after the surgery *B. breve* BBG-01 was administered as 0.5 mL (3.3×10^8 cfu) in sterile water. On day 10 the infant's gastric fluid became bilious and C-reactive protein (CRP) was elevated at 1.2 mg/dL, the white blood cell (WBC) count was $3500/\text{mm}^3$, with 18% bands and 26% neutrophils. Ampicillin/sulbactam and amikacin were initiated and enteral feedings were discontinued. On Day 12, the CRP and WBC values increased to 8.2 mg/dL and $9520/\text{mm}^3$, with 16% bands and 42% neutrophils. Ampicillin/sulbactam was switched to meropenem. Blood cultures taken on day 10 grew *Bifidobacterium* spp. and the oral probiotic therapy was discontinued. Polymerase chain reaction (PCR) analysis of the *Bifidobacterium* spp. in the blood cultures gave a positive indication of presence of *B. breve* and specifically *B. breve* BBG-01. A monoclonal antibody against *B. breve* BBG-01 gave a positive response as well. The patient eventually recovered without any sequelae or complications, according to the report authors.

v. Summary of Results from Infant Studies

B. breve M-16V has been administered to 430 health compromised and/or premature infants participating in multiple studies over a twenty year period (Bennet et al., 1992; Akiyama et al., 1994; Fujii et al., 2006; Hattori et al., 2003; Ishizeki et al., 2004; Li et al., 2004; Sato et al., 2003; Taniuchi et al., 2005; Umeda et al., 2010; van der Aa et al., 2010; van der Aa et al., 2011; van der Aa et al., 2012; Wang et al., 2007; Yamada et al., 2002) at doses up to 1.5×10^{10} cfu/d (Taniuchi et al., 2005) for durations up to 3 mo (Taniuchi et al., 2005; van der Aa et al., 2010; van der Aa et al., 2011; van der Aa et al., 2012). The Van der Aa study (reported on in three separate articles published in 2010, 2011, and 2012) supplemented infant formula with *B. breve* M-16V at a level of 1.3×10^9 cfu/100 mL; an infant consuming an average of 800 mL of supplemented formula per day would be receiving 10.4×10^9 cfu, or 1.04×10^{10} cfu per day. It is important to note that these levels of consumption of *B. breve* M-16V occurred in infants having both cow's milk hypersensitivity and atopic dermatitis (Taniuchi et al., 2005) and in infants having atopic dermatitis alone (van der Aa et al., 2010; van der Aa et al., 2011; van der Aa et al., 2012). Atopy-related endpoints were not negatively impacted in these studies, the formula was well tolerated, and no adverse effects were mentioned.

Two studies investigated the effects of other *B. breve* strains in very low birthweight premature infants (Kitajima et al., 1997 (two studies)). Kitajima and co-workers administered 3×10^9 cfu/d for 7 and 28 d (two studies), and reported mild functional ileus due to the corn starch present in the formulation (preliminary study) which was negated by suspending the bacterium in distilled water (second study by the same authors). Additionally, weight gain was increased between 4-8 wk of age in infants consuming *B. breve* YIT4010 (BBG) versus controls. The trend for increased weight persisted out to 18 mo.

Four studies administered *B. breve* strains in the presence of one or more additional strains; doses of the *B. breve* component ranged from 2×10^8 cfu/d to 9×10^9 cfu/d (Bennet et al., 1992; Kukkonen et al., 2008 (same study as Kekkonen, et al. 2006); Braga et al., 2011), and exposures ranged from 5 d to 6 mo. The Bennet study (1992) was the only one to utilize *B. breve* M-16V in combination with other bacteria. In these studies, growth metrics were equivalent and gastrointestinal issues occurred at equal rates across treatment and control groups. Studies of infants at high-risk for allergies did not indicate an adverse effect of *B. breve* on infection rates, respiratory parameters, vomiting, or ability to mount an immune response to various vaccines.

B. breve M-16V has been shown to be safe and well tolerated, and does not compromise growth in infants ingesting levels of up to 1.5×10^{10} cfu/d (Hattori, et al. 2003; Taniuchi et al., 2005; Van der Aa, et al. 2010, 2011, and 2012).

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Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<i>B. breve</i> M-16V				
Taniuchi et al. 2005 Highest dose tested: 1.5×10^{10} cfu/d Longest exposure: 3 mo Number participants receiving <i>B. breve</i> M-16V: 10	<i>Objective:</i> To evaluate whether oral administration of bifidobacteria influences the intestinal microbiota and allergic symptoms of infants with cow's milk hypersensitivity with atopic dermatitis. <i>Study type:</i> Randomized <i>Test article:</i> <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)	Seventeen infants (3.1-18.5 mo) with cow's milk hypersensitivity with atopic dermatitis and who had < 30% <i>Bifidobacterium</i> spp. in their intestinal microbiota were selected and randomly divided into two groups: 1) <i>B. breve</i> M-16V at 5 or 15×10^9 colony-forming units per day, given orally, for 3 mo ($n=10$) 2) no intervention ($n=7$) Infants were fed the casein-hydrolyzed formula new-MA-1 for at least 2 wks prior to receiving the bacteria. The bacterial preparation did not contain milk protein. Fecal microbiota analysis was carried out before, and at 1, 2, and 3 mo after the intervention. Allergic symptom scores were assessed, as well.	In group 1, the mean preadministration proportion of intestinal microbiota belonging to <i>Bifidobacterium</i> spp. was $10.62 \pm 14.8\%$. The proportion of <i>Bifidobacterium</i> spp. increased to 36.7 ± 25 ($P = 0.0173$), 35.19 ± 26.5 ($P = 0.0077$) and $35.54 \pm 28.6\%$ (statistics not reported) at 1, 2, and 3 mo, respectively. The control group did not experience an increase in the proportion of <i>Bifidobacterium</i> in the intestinal microbiota over the course of the study. In group 1, the cutaneous symptom scores at 1, 2, and 3 mo were significantly decreased ($p = 0.0255$, 0.0147 and 0.0378, respectively) versus the initial scores. However, cutaneous symptom scores were also reduced at 1 and 3 mo ($p = 0.0394$ and 0.0407) in the control group, versus initial scores. The total allergic score was significantly reduced at all time points in group 1 versus baseline levels ($p = 0.0156$, 0.0142, and 0.0359, respectively), whereas no reduction was observed in the control group.	The authors made no mention of adverse effects.

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Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Hattori et al. 2003 (English translation from Japanese) Highest dose tested: 1.5 × 10¹⁰ cfu/d Longest exposure: 1 mo Number participants receiving <i>B. breve</i> M-16V: 8	Objective: To study the effects of oral administration of <i>B. breve</i> M-16V on fecal microbiota and allergic symptoms in infants having allergic dermatitis. Study type: Controlled Test article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)	Fifteen infants (12M/3F; 8.8 ± 4.3 mo old) with atopic dermatitis and an intestinal microbiota that was deficient in <i>Bifidobacterium</i> species and who had been fed extensively hydrolyzed casein milk for at least two weeks were enrolled. Eight infants were administered <i>B. breve</i> M-16V 5 × 10 ⁹ or 1.5 × 10 ¹⁰ cfu/day for one month, and 7 infants served as controls. Changes in fecal microbiota and allergic symptoms were compared between the two groups. The extensively hydrolyzed casein milk was also supplemented with 0.13 g raffinose/100 mL milk.	The proportion of bifidobacteria present in the fecal flora were significantly increased at the end of the study in the treatment group, versus baseline levels (<i>p</i> <0.05). The proportion of total aerobic bacteria present also significantly decreased (<i>p</i> <0.05), while the total anaerobic proportion significantly increased (<i>p</i> <0.05), versus baseline. The cutaneous symptom score and the total allergic score were significantly reduced (<i>p</i> =0.0176 and 0.0117, respectively) at the end of the study in the <i>B. breve</i> M-16V group, but not in the control group. There was no correlation between the degree of allergic symptom improvement and changes in the fecal microbiota.	The authors made no mention of adverse effects.

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Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Van der Aa, et al. 2010 Highest dose tested: 1.0×10^{10} cfu/d based on a consumption of 800 ml/day Longest exposure: 12 wk Number participants receiving <i>B. breve</i> M-16V: 46	<p><i>Objective:</i> To investigate the effect of a synbiotic mixture of the severity of atopic dermatitis in infants.</p> <p><i>Study design:</i> Double-blind, placebo-controlled, multi-center</p> <p><i>Test article:</i> <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)</p>	<p>Ninety infants with atopic dermatitis, aged < 7 mo and exclusively formula fed, were assigned to receive either an extensively hydrolyzed formula containing <i>B. breve</i> M-16V (1.3×10^9 cfu/100 mL) and a galacto-/fructo-oligosaccharide mixture (Immunofortis®; 9:1 short chain GOS:long chain FOS, 0.8 g/100 mL) ($n=46$), or the same formula without synbiotics ($n=44$) for 12 wk. Primary outcome was the severity of atopic dermatitis, measured using the SCORAD index. Intestinal microbiota composition was a secondary outcome.</p>	<p>The severity of IgE-associated atopic dermatitis was significantly reduced in the synbiotic group versus the placebo group ($P=0.04$) at 12 wk. The amount of bifidobacteria, expressed as percent of the total fecal flora, was significantly increased in the synbiotic group, vs. placebo at wk 1 ($P=0.045$) and wk 12 ($P<0.001$). Percent of <i>Clostridium lituseburens</i> /<i>C. histolyticum</i> and <i>Eubacterium rectal/Clostridium coccoides</i> was significantly reduced in the fecal flora of the synbiotic group vs. control at 12 wk ($P=0.02$ and <0.001, respectively). Fecal pH, L-lactate, D-lactate, and acetic acid were decreased at 12 wk in the synbiotic group vs. placebo ($P=0.001$, <0.001, <0.001). Fecal butyric acid, isobutyric acid, and isovaleric acid increased at 12 wk in the synbiotic group vs. placebo ($P=0.04$, 0.02, 0.02). Fecal consistency was significantly reduced at all points measured throughout the study ($P=0.002$ for wk 1-4, $P=0.02$ for wk 5-8, $P=0.05$ for wk 9-12) in the synbiotic group vs. control. Episodes of dry stools, constipation, and diaper dermatitis during the study were also reduced in the treatment group vs. placebo ($P=0.001$, 0.01, 0.008).</p>	<p>Growth, weight, renal and liver function did not differ between the two groups. Diarrhea and gastroenteritis occurred equally in both groups, and there were no differences in parent-reported bowel cramps, flatulence and regurgitation. Adverse event incidence was similar between the groups. There were two serious adverse events in the synbiotic group: hospitalization due to respiratory syncytial virus bronchiolitis and severe cow's milk allergy. There were no serious adverse events in the control group. None of the reported adverse events were considered to be treatment-related by the authors. One child used antibiotics during the study in the synbiotics group and five children in the control group used antibiotics. The synbiotic mixture was reported to be well tolerated.</p>

000075

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Van der Aa et al. 2011 [NOTE: Same study as Van der Aa, et al. 2010, but different endpoints reported]</p> <p>Highest dose tested: 1.0×10^{10} cfu/d based on a consumption of 800 ml/day</p> <p>Longest exposure: 12 wk</p> <p>Number participants receiving <i>B. breve</i> M-16V: 46</p>	<p>Objective: To investigate the effect of early intervention with synbiotics on the prevalence of asthma-like symptoms in infants with atopic dermatitis.</p> <p>Study type: Double-blind, placebo-controlled, multi-center</p> <p>Test Article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)</p>	<p>Ninety infants with atopic dermatitis, aged < 7 mo, were randomized to receive an extensively hydrolyzed formula containing <i>B. breve</i> M-16V (1.3×10^9 cfu/100 mL) and a galacto-/fructo-oligosaccharide mixture (Immunofortis®; 9:1 short chain GOS:long chain FOS, 0.8 g/100 mL) ($n=46$), or the same formula without synbiotics ($n=44$) for 12 wk. After 1 yr, the prevalence of respiratory symptoms and asthma medication use was evaluated, and total serum IgE and specific IgE against aeroallergens were determined. Seventy-five children completed the 1 yr follow-up evaluation (70.7% male, mean age 17.3 mo). Thirty-six infants in the synbiotics group were analyzed at follow-up and 39 infants were analyzed in the control group.</p>	<p>Six infants discontinued the synbiotic formula: one refused formula; three did not show up at appointments; one used other formula; and one had intercurrent disease. Four infants were lost to follow up in this group due to not being able to come to the hospital. Two infants discontinued the control formula: one refused the formula; and one used antihistamine medication (protocol violation). Three infants were lost to follow up in the control group for the same reasons as the synbiotic group.</p> <p>Prevalence of asthma-like symptoms was decreased in the synbiotic group, versus control, at the 1 yr follow-up, including: frequent wheezing ($p=0.04$); wheezing apart from colds ($p=0.056$); wheezing and/or noisy breathing apart from colds ($p=0.001$); asthma medication use ($p=0.049$); and reduced new use of asthma medication at follow-up vs. baseline ($p=0.02$).</p> <p>In a subgroup of children who were IgE-negative at baseline, the change in total serum IgE concentration from baseline to 1 yr follow-up was reduced in the synbiotic group versus placebo group ($P=0.04$). The</p>	<p>The authors made no mention of adverse effects.</p>

000006

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
			<p>percentage of children having elevated specific IgE against cat was significantly lower in the synbiotic group versus control group ($P=0.03$). However, more children had cats as pets in the control group versus the synbiotic group at the 1 yr follow-up. The number of children who had newly elevated specific IgE against cat at 1 yr was higher ($P=0.053$) in the control group (5/33) versus the synbiotic group (0/29). None of the IgE positive children had cats in the home.</p>	
<p>Van der Aa et al., 2012 [NOTE: Same study as Van der Aa, et al. 2010, but different endpoints reported] Highest dose tested: 1.0×10^{10} cfu/d based on a consumption of 800 ml/day Longest exposure: 12 wk Number participants receiving <i>B. breve</i> M-16V: 46</p>	<p>Objective: To determine the effects of <i>B. breve</i> M-16V combined with short chain galacto-oligosaccharides and long chain fructo-oligosaccharides on atopic markers, <i>ex vivo</i> cytokine production by peripheral blood mononuclear cells (PBMCs) and circulating regulatory T cell percentage in infants with atopic dermatitis. Study type: Double-blind, placebo-controlled, randomized, multi-center</p>	<p>Ninety full-term infants aged 0-7 mo diagnosed with atopic dermatitis and exclusively formula fed at the time of enrollment were included in the study. Exclusion criteria included presence of major medical problems and use of probiotics, systemic antibiotics or anti-mycotics or immunosuppressive drugs during the prior 4 wk. Infants were randomized to receive either an extensively hydrolyzed, whey-based formula (Nutrilon Pepti; Nutricia, Zoetermeer, the Netherlands) ($n=44$) or the same formula with 1.3×10^9 cfu <i>B. breve</i> M-16V/100 mL plus 0.8 g of 90% scGOS/10% lcFOS per 100 mL formula ($n=46$) for 12 wk. Blood samples were taken at baseline and at 12 wk for analysis of various immune parameters.</p>	<p>No statistically significant effects from the synbiotic-containing formula were noted for plasma levels of IL-5, IgG1, IgG4, cutaneous T cell attracting chemokine (CTACK), or thymus and activation-regulated chemokine (TARC). There were also no effects on <i>ex vivo</i> cytokine production by peripheral blood mononuclear cells (PBMCs) stimulated with anti-CD3/anti-CD28 in the synbiotic group for IL-4, IL-5, IL-13, IL-6, IL-12p40p70, IFN-γ, IL-17, IL-10, and TGF-β. There were no significant differences between groups in the percentages of CD3+, CD4+CD8- and FoxP3+CD25+ cells at either baseline or at 12 wk. The mean percentage of FoxP3+CD25+CD127- (Tregs)</p>	<p>The authors made no mention of adverse effects.</p>

000077

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
	<p>Test Article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)</p>		<p>cells did not differ between the groups. In PBMCs stimulated with egg, there was a statistically significant between the groups for IL-12p40/70, IL-12p70 cytokine levels ($P = 0.04$ and 0.01, respectively). In peanut-stimulated cells there was a difference in IL-12p70 levels between the groups ($P=0.003$). The clinical significance of these results is unclear.</p> <p>The authors did not find a beneficial effect of the synbiotic formula on the severity of infant atopic dermatitis, eosinophilic granulocyte count or serum IgE levels at 12 wk. There was a beneficial effect of synbiotics on the severity of atopic dermatitis in the subgroup of infants with IgE-associated atopic dermatitis.</p>	

82000078

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
De Kivit et al., 2012 [NOTE: Same study as Van der Aa, et al. 2010, but different endpoints reported] Highest dose tested: Approximately 1.0×10^{10} cfu/d based on the consumption of 800 ml/day Longest exposure: 12 wk Number participants receiving <i>B. breve</i> M-16V: 46	Objective: To study the effect of <i>B. breve</i> M-16V combined with short chain galacto-oligosaccharides and long chain fructo-oligosaccharides on galectin-9 serum levels and peripheral blood monocyte cell responses to galectin-9. Test Article: <i>B. breve</i> M-16V combined with short chain galacto-oligosaccharides and long chain fructo-oligosaccharides on	Ninety full-term infants aged 0-7 mo who were diagnosed with atopic dermatitis and were exclusively formula fed at the time of enrollment were included in the study. Infants were randomized to receive either an extensively hydrolyzed, whey-based formula (Nutrilon Pepti; Nutricia, Zoetermeer, the Netherlands) ($n=44$) or the same formula with 1.3×10^9 cfu <i>B. breve</i> M-16V/100 mL plus 0.8 g of 90% scGOS/10% lcFOS per 100 mL formula ($n=46$) for 12 wk. Blood samples were taken at baseline and at 12 wk for analysis of galectin-9 serum levels and response of peripheral blood mononuclear cells to varying levels of added galectin-9 <i>in vitro</i> .	Serum galectin-9 levels were significantly higher at 12 wk, versus baseline levels, in the treatment group ($P<0.01$). Peripheral blood mononuclear cells isolated at 12 wk from the treatment group exhibited a dose-related decrease in IL-17 secretion <i>in vitro</i> when exposed to various doses of galectin-9. The clinical significance of these results is unclear.	

620000

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

References	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Bennet, et al. 1992 Highest dose tested: 9 × 10⁹ cells/d Longest exposure: 5 d	Objective: To determine whether orally administered bifidobacteria and/or lactobacilli could be cultured from infant feces after antibiotic treatment. Test article: <i>Bifidobacterium breve</i> BB-576 (also known as <i>B. breve</i> M-16V; Morinaga Milk Industry Co., Ltd), <i>Bifidobacterium longum</i> BB-536, <i>Lactobacillus acidophilus</i> , LAC-343	Doses of 3 × 10 ⁹ cells of one strain or a mixture of all three strains at 3 × 10 ⁹ cells each were fed 3×/d (total = 9 × 10 ⁹ cells) at mealtimes to 11 infants aged 0-8 wk. Bacteria were fed beginning on the first day after stopping antibiotic treatment and this was continued for 5 d. Bacterial species were isolated from fecal specimens taken on the last day of bacteria dosing, and 5 and 15 d afterwards. Fecal samples were also taken from three control infants who did not receive these bacterial preparations. Infants received either <i>B. breve</i> , strain BB-576 (n=3), <i>B. longum</i> , strain BB-536 (n=3), <i>L. acidophilus</i> (n=3), a mix of all three (n=2), or no intervention (n=3).	<i>B. breve</i> was recovered from 3/3, 2/3, and 0/1 fecal samples taken from infants receiving only <i>B. breve</i> BB-576 on the last day of bacterial dosing, 5, and 15 days afterwards, respectively.	The authors reported that no side effects were noted.
Fujii, et al. 2006 Highest dose tested: 2 × 10⁹ cfu/d Longest exposure: 28 d Number participants receiving <i>B. breve</i> M-16V: 11	Objective: To examine the effect of <i>B. breve</i> M-16V on the immune system of preterm infants, especially as relates to TGF-β signaling. Study type: Randomized, controlled Test article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)	Nineteen preterm infants admitted to a neonatal intensive care unit were randomized to receive either 1) 0.5 mL of 1.0 × 10 ⁹ cells of <i>B. breve</i> M-16V in 5% glucose solution 2×d nasogastrically, beginning several h after birth, for 28 d (n=11; 7M/4F; mean birth weight 1378 ± 365 g; mean gestational age 31.3 ± 3.16 wk) or 2) vehicle supplementation (n=8; 5M/3F; 1496 ± 245 g; 31.2 ± 1.98 wk). Blood samples were collected on d 0, 14, and 28 for ELISA. Infants having chromosomal or congenital anomalies, history of intrauterine infection or surgery, or whose mothers had received corticosteroid treatment were excluded.	TGF-β levels were elevated in both groups at d 14. By d 28, serum TGF-β levels were significantly greater in the treatment group versus the control group (P = 0.005). No significant differences were observed between control and treatment groups for expression of Smad2, Smad4, or Smad7 mRNA. Smad3 mRNA expression was higher in the <i>B. breve</i> M-16V group versus the control on d 28 (P=0.03).	The authors report that no adverse effects were observed after <i>B. breve</i> supplementation.

080000

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Sato, et al. 2003 (English translation summary; article in Japanese)</p> <p>Highest dose tested: 1×10^9 cfu/d</p> <p>Longest exposure: Until 36 wk of corrected gestational age</p> <p>Number participants receiving <i>B. breve</i> M-16V: 75</p>	<p>Objective: To study the effects of administration of <i>B. breve</i> on infection in premature infants.</p> <p>Study type: Controlled</p> <p>Test article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)</p>	<p>One hundred ninety infants with a birth weight < 1.5 kg were considered for inclusion in the study. Infants were excluded because of death or hospital transfer within 3 d after birth. One hundred sixty-two subjects were enrolled. 75 infants received 1×10^9 cfu <i>B. breve</i> M-16V (28.3 ± 3.1 wk mean gestational age; 1027.3 ± 279.7 g mean birth weight) at the initiation of tube feeding and 87 infants served as controls (28.6 ± 2.6 wk; 1064.0 ± 275.0 g). Infants were fed until 36 wk of corrected gestational age. Occurrence of infection was defined as an increase in C-reactive protein to ≥ 2 mg/dL or < 2 mg/dL if infection was suspected based on blood test results, clinical symptoms, or other findings. Infants were excluded if they had increased C-reactive protein ≥ 2 mg/dL within 72 h of birth.</p>	<p>Infection developed in 20/75 infants in the treatment group (26.6%) and 33/87 infants in the control group (37.9%).</p> <p>Enteral feeding reached 100 mL/kg/d at 15.3 d in the treatment group, versus 19.8 d in the control group.</p> <p>Mean duration of hospitalization was 89.6 d in the treatment group and 102.3 d in the control group.</p>	<p>Mean body weights on the calculated date of confinement (estimated delivery date) were 2426.1 and 2084.0 g in the treatment and control groups, respectively. The authors made no mention of adverse events.</p>
<p>Yamada, et al. 2002 (English summary translation from Japanese)</p> <p>Highest dose tested: 1×10^9 cfu/d</p> <p>Longest exposure: 5 d</p> <p>Number participants receiving <i>B. breve</i> M-16V: 133</p>	<p>Objective: To determine whether <i>B. breve</i> M-16V could control methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) infection in a neonatal intensive care unit (NICU).</p> <p>Study type: Controlled</p> <p>Test article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)</p>	<p>Two hundred sixty-six infants were admitted to a NICU and were enrolled in the study. Infants having chromosomal aberrations were excluded. Every other subject was given 1×10^9 cfu of <i>B. breve</i> M-16V as powder dissolved in 2 mL of milk for 5 d starting from the time milk was given. Samples were taken for culture and detection of MRSA in infants at admission and days 7 and 14 after admission. Thereafter, cultures were done every 1-2 weeks, depending on presence of MRSA.</p>	<p>Prior to <i>B. breve</i> M-16V administration, MRSA carrier rate was 30.5% of admitted patients and incidence of MRSA infection and neonatal toxic shock syndrome (TSS)-like exanthematous disease (NTED) was 9.5%. In the study, after administration of <i>B. breve</i> M-16V, the MRSA carrier rate was 12.3% and the incidence of MRSA infection and NTED was 2.2%.</p>	<p>Detection rate of <i>Pseudomonas aeruginosa</i> was 3.9% prior to administration of <i>B. breve</i> M-16V and 14.6% afterwards. The clinical significance of this was not discussed in the translated summary that was provided.</p>

000081

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Akiyama, et al. 1994</p> <p>Highest dose tested: 5×10^8 cells/d</p> <p>Longest exposure: 8 wk</p> <p>Number participants receiving <i>B. breve</i> M-16V: 5</p>	<p>Objective: To study the effects of administering <i>B. breve</i> on the intestinal microbiota of extremely premature infants.</p> <p>Test article: <i>B. breve</i> biovar <i>a</i> (also known as <i>B. breve</i> M-16V; Morinaga Milk Industry Co., Ltd)</p>	<p>Very low birth weight infants with birth weights < 1250 g were enrolled. Infants having a deformity, chromosomal abnormality, or intrauterine infection were excluded. Infants were randomly assigned to receive either <i>B. breve</i> preparation (<i>n</i>=5) or dextrin (<i>n</i>=5). All subjects were on artificial ventilation except one infant in the control group. Bacterial preparation (0.5 g containing 5×10^8 cells) or dextrin was dissolved in 1 mL of sterile water and administered intragastrically 1×/d until 8 wk of age. Fecal samples were collected by rectal stimulation at 1, 2, 4, 6, and 8 wk after birth.</p>	<p>Administered <i>B. breve</i> biovar <i>a</i> was recovered in the feces of infants throughout the study. This biovar was also observed in fecal samples assayed in the control group.</p>	<p>The authors mention that the preparation was used without any clinical problem.</p>
<p>Ishizeki, et al. 2004 (English translation summary; article in Japanese)</p> <p>Highest dose tested: 5×10^8 cfu/d</p> <p>Longest exposure: 6 wk</p> <p>Number participants receiving <i>B. breve</i> M-16V: 13</p>	<p>Objective: To study the effects of administering various bifidobacteria preparations on the intestinal flora of low birth weight infants.</p> <p>Study type: Controlled</p> <p>Test article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd); <i>Bifidobacterium infantis</i> M-63 and <i>B. longum</i> BB536 were also used in the study.</p>	<p>Infants with birth weights ≥ 1000 g and < 200 g who were able to start enteral nutrition within 1 wk of birth were studied. Infants were studied in groups at different time periods, so this was not a concurrently running study. Treatments were either: none (control group; <i>n</i>=16; mean gestational week 30 wk, mean birth weight 1335 g); single species of <i>Bifidobacterium</i> (<i>B. breve</i> M-16V; <i>n</i>=15; 30 wk, 1282 g); or three species of <i>Bifidobacterium</i> (<i>B. breve</i> M-16V + <i>B. infantis</i> M-63 + <i>B. longum</i> BB536; <i>n</i>=13; 30 wk, 1247 g). Bifidobacteria were administered for 6 wk beginning with the initiation of enteral nutrition. Fecal samples were collected after 1, 2, 4, 6, and 8 wk of treatment. Samples were analyzed within 24 h of collection.</p>	<p>Rates of detection of bifidobacteria at 1 wk post-administration were 14, 57, and 100% in the control, one-species group and three-species group, respectively. By wk 8, the rates were 27 and 90% in the one-species and three-species groups, respectively (control group data not given). In the three-species group, <i>B. breve</i> and <i>B. infantis</i> were detected in $\geq 85\%$ of the subjects and <i>B. longum</i> was detected in $\leq 40\%$ of the subjects over the entire administration period.</p>	<p>The authors made no mention of adverse effects.</p>

000082

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Umeda, et al. 2010 (English translation summary; article in Japanese) Highest dose tested: 5×10^8 cfu/d Longest exposure: 16 d Number participants receiving <i>B. breve</i> M-16V: 49	Objective: To study the effects of probiotics on extremely low birth weight infants. Test article: <i>B. breve</i> M- 16V (Morinaga Milk Industry Co., Ltd)	One hundred thirty-three extremely low birth weight infants were divided into a control group ($n=84$) and a treatment group ($n=49$). Control and probiotic groups had average gestational ages of 25.7 ± 2.1 and 25.7 ± 1.6 wk, and birth weights of 749.2 ± 164.0 g and $769.8 \pm$ 165.9 g, respectively. The treatment group received 5×10^8 cfu of <i>B. breve</i> M- 16V in water per day. Infants in the treatment group were exposed to <i>B. breve</i> M-16V for an average of 16.0 d and infants in the control group participated in the study for an average of 20.9 d. In the treatment group, 23 of 49 infants (46.9%) received fluconazole and zero of 89 infants in the control group received fluconazole.	Breast milk was started on days 3.3 and 1.8 (averages) for the control and probiotics group, respectively. Consumption of 100 mL/kg/day of milk occurred on d 20.9 in the control group and 16.0 d in the treatment group. The average time needed for infants to return to birth weight was 29.7 and 24.3, respectively ($p<0.05$).	NEC occurred in 9 infants (10.7%) in the control group and one infant (2.0%) in the treatment group ($p=0.06$). Death by NEC occurred in 5 infants (5.6%) in the control group and no infants in the probiotic group. Delayed sepsis (positive on blood culture) was observed in 28 infants (33.3%) in the control group and 4 infants (8.2%) in the treatment group ($p<0.01$). Fungal sepsis occurred in 3 infants in the control group and none in the treatment group.

000083

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcomes	Safety-Related Observations
Wang, et al. 2007 Highest dose tested: 3.2×10^8 cfu/d Longest exposure: 4 wk Number participants receiving <i>B. breve</i> M-16V: 33	<p>Objective: To investigate the effects of oral administration of <i>B. breve</i> M-16V on fecal lactic acid and short-chain fatty acids in low birth weight infants.</p> <p>Study type: Controlled</p> <p>Test article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)</p>	Sixty-six premature infants were enrolled in the study. Those having malformations, chromosomal abnormalities or intrauterine infections were excluded. Infants were divided into 3 groups by birth-weight: 22 extremely low birth-weight infants (<1000 g), 22 very low birth-weight infants (<1500 g) and 22 low birth-weight infants (<2500 g). Within each group, infants received either <i>B. breve</i> M-16V (1.6×10^8 cells in 0.5 mL of 5% glucose in sterile distilled water, administered intragastrically, 2x/d; n=11 per birth-weight group) or no intervention (n=11 per birth-weight group) until discharge. Breast milk was replaced with infant formula when necessary. Fecal samples were collected at 0, 2, and 4 wk after birth.	The ratio of fecal acetic acid to total short-chain fatty acids (SCFAs) was significantly higher in all treatment groups (due to the decrease in butyric acid), versus controls ($p < 0.05$) by 4 wk after birth. In general, fecal propionic and butyric acid levels decreased in the treatment groups versus control groups (not statistically significant) by 4 wk. Supplementation of <i>B. breve</i> M-16V decreased individual and total SCFAs in the extremely low birth weight infants at 4 wk, versus controls (not statistically significant except for butyric acid, $p < 0.05$), whereas results were less robust in higher birth weight infants. Fecal excretion of total SCFAs increased with birth weight and time.	The authors state that no adverse effects were observed after <i>B. breve</i> supplementation. No serious infections or positive blood cultures occurred; nor were C-reactive protein levels elevated.

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Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Li, et al. 2004 Highest dose tested: 3.2×10^8 cfu/d Longest exposure: 7 wk Number participants receiving <i>B. breve</i> M-16V: 10	Objective: To examine the health effects of early administration of bifidobacteria in low birth weight infants. Study type: Controlled, randomized Test article: <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd)	Low birth weight infants (avg birth weight 1489 g) were divided into three groups: A) 1.6×10^8 cells of <i>B. breve</i> M-16V, administered intragastrically with feeding, as a suspension in 0.5 mL of 5% glucose sterile distilled water, 2×/d, initiated within several hours after birth ($n=10$; mean 7.2 h) B) 1.6×10^8 cells of <i>B. breve</i> M-16V 2×/d, initiated within 24 h after birth ($n=10$; mean 36.5 h) C) no supplementation ($n=10$) Infants having deformities, chromosomal abnormalities or intrauterine infection were excluded. Gestational ages, birth weights and other clinically relevant parameters were similar across groups. Treatments were administered for 7 wk. Fecal samples were collected daily for the first 2 wk after birth and then weekly thereafter up to 7 wk.	<i>Bifidobacterium</i> spp. were detected by 3.4 ± 2.2 d in group A, and by 7.2 ± 3.8 d in group B ($P<0.05$). <i>Enterobacteriaceae</i> spp. were significantly lower in group A versus groups B and C at 2 wk ($P<0.05$), but this difference disappeared after 2 wk.	The authors state that they did not observe any side effects due to the administration of <i>B. breve</i> . There were no significant differences in the incidence of NEC, infectious disease or sepsis among the three groups, according to the authors. No symptoms were observed that could be attributed to the administration of <i>B. breve</i> .

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Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<i>B. breve</i>				
Kitajima, et al. 1997 Highest dose tested: 3×10^9 cfu/d and 0.5×10^9 cfu/d Longest exposure: 7 d and 28 d, respectively Number participants receiving <i>B. breve</i>: 66 @ 3×10^9 cfu/d 45 @ 0.5×10^9 cfu/d	<i>Objective:</i> To investigate the colonization of the bowels of very low birth weight infants with <i>B. breve</i> . <i>Study type:</i> Preliminary open-label study and randomized, controlled trial <i>Test article:</i> <i>B. breve</i> YIT4010 (BBG) was supplied as a freeze-dried powder in corn starch, containing 10^9 colony-forming units per g dry weight and was from Yakult Honsya Co. Ltd., Tokyo, Japan.	<i>Preliminary study:</i> Sixty-six preterm infants were enrolled and administered 1 g of <i>B. breve</i> YIT4010 (BBG) powder (10^9 colony-forming units) up to 3x/d (variable dosing). Sixteen infants (28.3 ± 2.9 wk gestation; 1052 ± 328 g) were given <i>B. breve</i> YIT4010 within 7 d of life and for a duration of up to 7 d (median 5 d, range 1-7 d). Fifty infants were given <i>B. breve</i> YIT4010 after 7 d of life and for at least 7 d (median 14 d, 7-48 d). <i>Randomized, controlled study:</i> Ninety-seven very low birth weight infants were enrolled in the study; 91 were followed up for 2 mo after birth and 70 were followed up for 3 yr to examine their growth. Inclusion criteria included birth weight of < 1500 g and exclusion criteria included infants having major anomalies, severe asphyxia and severe intrauterine growth retardation. Infants were randomly allocated to receive 1 mL of the supernatant of <i>B. breve</i> YIT4010 suspension with distilled water containing $\sim 0.5 \times 10^9$ live cells ($n=45$) or distilled water as control ($n=46$). The first dose was given within the first 24 h of life, then once a day for 28 d. Meconium and stools were collected for anaerobic culture once per week \times 8 wk.	<i>Preliminary study:</i> Of the fifty infants receiving <i>B. breve</i> YIT4010 after 7 d of life, two extremely preterm infants had mild functional ileus, as evidenced by undigested corn starch aggregates in their stool. <i>Randomized, controlled study:</i> Due to the adverse effect of corn starch aggregates observed in the preliminary study, <i>B. breve</i> YIT4010 was administered as the supernatant of a suspension of the bacterial preparation in distilled water. Colonization rates of infants were higher in the treatment group, versus control, across all gestational age groupings (no statistics). Treated infants had decreased aspirated air volume in stomach, vs. controls, on days 23-28 ($P<0.05$). <i>B. breve</i> YIT4010 infants had higher feeding volumes ($P<0.05$) after 10 d, versus control infants, and higher body weight ($P<0.05$) after 4 wk, versus control infants. Infants that had viable <i>B. breve</i> YIT4010 in the feces required fewer doses of indomethacin, versus infants that that did not ($p= 0.0621$).	<i>Preliminary study:</i> There were no other adverse effects from <i>B. breve</i> YIT4010 itself, aside from the two incidents of mild functional ileus. <i>Randomized, controlled study:</i> Two infants died at 5 and 16 d of age, respectively; 3 infants were transferred back to the referring hospital before they were 3 wk old; one girl was excluded due to diagnosis of coarctation of aorta at 15 d of age. The authors mention that bifidobacteria cannot produce vitamin K, so infants that consume <i>Bifidobacterium</i> should be checked for vitamin K deficiency.

000089

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<i>B. breve</i> Administered in Combination with Other Probiotics				
Kukkonen, et al. 2006 [*NOTE: Different endpoints, same study as that reported on in Kukkonen, et al. 2008] Highest dose tested: 2×10^8 cfu/d (<i>B. breve</i> Bbi99 as mixture with other bacteria) Longest exposure: 6 mo Number participants receiving <i>B. breve</i>: 967	Objective: To study the effect of probiotics on vaccine antibody responses in six-month-old infants. Study type: Randomized, placebo-controlled, double-blind Test article: <i>B. breve</i> Bbi99 was administered as a mixture with <i>Lactobacillus rhamnosus</i> GG, <i>L. rhamnosus</i> LC705, and <i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> JS. Probiotics were from Valio Limited, Helsinki, Finland	The study comprised a sub-population derived from 1223 infants randomized to an allergy-prevention trial on high-risk infants. Eligible infants had at least one parent with doctor-diagnosed atopic disease. Premature infants and those having major malformations were excluded. Infants were randomized to either the probiotic ($n=47$) or control ($n=40$) group prior to birth. Four wk prior to delivery, mothers took one capsule of either probiotics or placebo twice daily. Each probiotics capsule contained 5×10^9 colony-forming units (cfu) <i>Lactobacillus rhamnosus</i> GG + 5×10^9 cfu <i>L. rhamnosus</i> LC705 + 2×10^8 cfu <i>B. breve</i> Bbi99 + 2×10^9 cfu <i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> JS. Placebo capsules contained microcrystalline cellulose only. After birth, infants received the contents of one opened capsule plus 20 drops of sugar syrup with (probiotics) or without (placebo) 0.8 g of galacto-oligosaccharides daily for 6 mo. Infants were vaccinated against diphtheria and tetanus toxoid, and <i>Bordetella pertussis</i> cells at 3, 4, and 5 mo. Either Hiberix® or HibTITER® vaccine (both for <i>Haemophilus influenzae</i> type b, Hib) was also given at 4 mo.	Differences in the mean concentrations of anti-diphtheria IgG and of anti-tetanus IgG were not significant between the study groups. In all cases, diphtheria and tetanus IgG titers exceeded the protective level of 0.01 UI/mL. The mean anti-Hib IgG concentration was higher in the probiotic group versus the placebo group, but this difference was not statistically significant. Anti-Hib antibodies were detectable in 66% of infants in the probiotic group and 48% of the placebo group ($p = 0.174$) and protective ($\geq 1 \mu\text{g/mL}$) anti-Hib IgG antibodies occurred in the probiotic group (50%) more frequently versus the placebo group (21%) ($p = 0.020$). Samples drawn from non-compliant infants and those having an unsuitable time window between vaccination and blood sampling were excluded.	The authors state that they observed no important side effects during the treatment. Growth and abdominal symptoms were similar between the groups. Probiotic supplementation in infants at high risk of atopy did not interfere with antibody responses to the study vaccines.

000087

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcomes	Safety/Adverse Events
<p>Kukkonen, et al. 2008 [NOTE: Same study as that reported on in Kukkonen, et al. 2006, but different endpoints reported]</p> <p>Highest dose tested: 2×10^8 cfu/d</p> <p><i>Bifidobacterium breve</i> Bbi99 plus other bacterial strains</p> <p>Longest exposure: 4 wk (pregnant mothers) 6 mo (infants)</p> <p>Number participants receiving <i>B. breve</i>: 967</p>	<p>Objective: To study the safety and long-term effects of feeding synbiotics to newborn infants.</p> <p>Study type: Randomized, placebo-controlled, double-blind</p> <p>Test article: <i>B. breve</i> Bb99 was given in conjunction with <i>L. rhamnosus</i> GG and LC705, <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS. Probiotics were from Valio Limited, Helsinki, Finland</p>	<p>1223 pregnant mothers carrying infants at high risk for allergy were enrolled and assigned to receive capsules containing 5×10^9 colony-forming units (cfu) <i>L. rhamnosus</i> GG + 5×10^9 cfu <i>L. rhamnosus</i> LC705 + 2×10^8 cfu <i>B. breve</i> Bbi99 + 2×10^9 cfu <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS or placebo capsules that contained microcrystalline cellulose only. Mothers consumed capsules twice daily for 4 wk prior to delivery. After birth, infants received the contents of one opened capsule plus 20 drops of sugar syrup with (probiotics) or without (placebo) 0.8 g of galacto-oligosaccharides (bovine origin) daily for 6 mo. The follow up period lasted from 6-24 mo. Exclusion criteria included birth at < 37 wk gestation, being a B twin (2nd born), and major malformations. Infants were examined at 3, 6 and 24 mo and parents completed questionnaires at 3, 6, 12, and 24 mo. A total of 446 infants in the synbiotic group and 456 infants in the control group completed the study.</p>	<p>Of the 1223 randomized mothers, 156 refused to participate and 49 of their infants, plus 14 B twins, were ineligible. Of these, 8 infants in the synbiotic group and 7 in the placebo group were born prematurely.</p> <p>At 6 mo, there were 506 infants in the synbiotic group and 512 in the control group. More infants in the control group discontinued the intervention at 6 mo due to infection (24), oxygen supplementation (18), vomiting (7) or respiratory infection requiring hospitalization (15), versus the intervention group (11, 11, 4, and 8, respectively).</p> <p>During the follow-up period, respiratory infections occurred less frequently in the synbiotic group than in the placebo group ($P=0.023$) and the total number of respiratory infections was lower in the synbiotic group ($P=0.009$). Middle ear infections tended to be lower in the synbiotic group ($P=0.068$) but gastroenteritis was equally common in both groups.</p>	<p>There were no statistically significant differences between the groups for length, weight or head circumference at 6 and 24 mo. Normal growth was reported for both groups.</p>

000088

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Braga, et al. 2011 Highest dose tested: 3.5×10^9 cfu/d mixture of <i>Bifidobacterium breve</i> plus <i>Lactobacillus casei</i> Longest exposure: 28 d Number participants receiving <i>B. breve</i>: 122	<p><i>Objective:</i> To assess whether probiotics could prevent the occurrence of NEC stage ≥ 2 by the criteria of Bell in very low birth weight preterm infants.</p> <p><i>Study type:</i> Double-blind, randomized, controlled</p> <p><i>Test article:</i> <i>B. breve</i> and <i>Lactobacillus casei</i> were from Yakult, São Paulo, Brazil</p>	Two hundred fifty-eight preterm infants weighing 750-1499 g who were admitted to the neonatal intensive care unit during the study period were enrolled, and 243 were randomized to receive either human milk plus supplementation with 3.5×10^7 to 3.5×10^9 cfu of a mixture of <i>L. casei</i> and <i>B. breve</i> ($n=122$) or human milk only ($n=121$). Three deaths occurred in the probiotic group before beginning the intervention; similarly, there were 9 deaths in the control group. Therefore, there were 119 infants in the probiotic group and 112 in the control group. The intervention began on d 2 of life and continued until d 30. None of the infants had major congenital malformations, previously diagnosed life-threatening chromosomal alterations or congenital infections diagnosed at birth. The primary outcome was the occurrence of NEC stage ≥ 2 as defined by Bell's modified criteria.	No cases of NEC stage ≥ 2 by Bell's criteria occurred in the synbiotics group; four cases occurred in the control group. There was a significant decrease in the amount of complete transition time of orogastric feeding tube to breastfeeding and in time to reach full enteral feeding in the probiotics group vs. the control ($P=0.03$ and 0.02 , respectively).	The authors observed no difference in the occurrence of sepsis or in the number of deaths between the probiotics and control group during the study.

680000

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Case Reports of Opportunistic <i>B. breve</i> Infections				
Nakazawa, et al. 1996	Case report of neonatal meningitis caused by <i>B. breve</i>	Second case report that implicates <i>B. breve</i> as the causative agent of neonatal meningitis. Patient was male, delivered by caesarian section at the 38 th gestational week, with birth weight of 2640 g. Symptoms include fever of up to 39°C, poor feeding, increased white blood cell count, and mononuclear cell dominant pleocytosis in the cerebrospinal fluid (CSF). Anaerobic Gram-positive rods were detected in a blood culture on the 4 th day after admission. The fever recurred 5 days after discontinuing antibiotics (ampicillin and cefotaxime) and anaerobic culture of the CSF indicated presence of the same Gram-positive bacterium as before. The same antibiotic therapy was restarted and gamma-globulin infusions were added.	The patient was completely cured after two relapses of meningitis and there were no neurological sequelae. The bacterium was identified as <i>B. breve</i> by gas-liquid chromatography. Route of infection was unclear.	Patient had good clinical course, in spite of relapse. Serum C-reactive protein was consistently negative, indicating low levels of inflammation.

060000

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Ohishi, et al. 2010</p> <p>Dose administered: 3.3×10^8 cfu/d <i>Bifidobacterium breve</i> BBG-01</p> <p>Exposure time: 8 d</p>	<p>Case report of neonatal sepsis caused by <i>B. breve</i> administered as probiotic therapy.</p> <p><i>Test article:</i> <i>B. breve</i> BBG-01 was from Yakult Honsya Co. Ltd, Tokyo, Japan</p>	<p>A female infant diagnosed with omphalocele at 13 wk gestation was delivered at 37 wk and 2 d of gestation by cesarean delivery. Birth weight was 2060 g and the liver and intestine were prolapsed. The infant also had polydactyly of the right hand. Four hours after birth, surgery to correct omphalocele was performed. Two days after the surgery, <i>B. breve</i> BBG-01 was administered as 0.5 mL (3.3×10^8) of the supernatant from mixing 1 g powdered bacterium (10^9 cfu/g) in 1.5 mL sterile water and centrifuging in a sterile environment. A peripheral arterial catheter was removed on d 8 and by d 10, gastric fluid became bilious. C-reactive protein (CRP) was elevated (1.2 mg/dL), white blood cell (WBC) count was $3500/\text{mm}^3$, with 18% bands and 26% neutrophils. Ampicillin/sulbactam and amikacin were initiated and enteral feedings were discontinued. On d 12, CRP and WBC count increased to 8.2 mg/dL and $9520/\text{mm}^3$, with 16% bands and 42% neutrophils. Ampicillin/sulbactam was switched to meropenem. Blood cultures taken on d 10 grew <i>Bifidobacterium</i> spp. and oral probiotic therapy was discontinued.</p>	<p>Polymerase chain reaction (PCR) analysis of the <i>Bifidobacterium</i> spp. isolated from the blood cultures was positive for <i>B. breve</i>, and <i>B. breve</i> BBG-01. Additionally, a monoclonal antibody against <i>B. breve</i> BBG-01 gave a positive response in the isolates.</p>	<p>The patient recovered without any sequelae or complications.</p>

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 00091

Table 14. Human Clinical Studies of *Bifidobacterium breve* – Infants

Reference	Objective/Study Type/ Test Article	Study Design	Outcome	Safety-Related Observations
Hata, et al. 1988	Case report of meningitis due to <i>Bifidobacterium</i> in an infant.	First case report of meningitis in an infant that was demonstrated to be caused by <i>Bifidobacterium breve</i> (<i>B. breve</i>). A 37 d old Japanese male infant who was delivered vaginally at 37 wk gestation (2840 g birth weight) to a mother with Behçet's disease developed an axillary temperature of 38.3°C, with a slightly distended anterior fontanelle. Rectal temperature was 38.6°C but the remainder of the physical examination was unremarkable. The infant was well-nourished and active. White blood cell count was elevated and the cerebrospinal fluid (CSF) contained 74 white blood cells (WBC)/mm ³ . CSF culture grew Gram-positive rods after 24 h, but blood culture was sterile. Intravenous cefotaxime (10 d) was initiated upon admission. After d 3 of antibiotic, the fever went away and the anterior fontanelle was flat. Fever recurred on the 12 th hospital day and CSF contained 633 WBCs/mm ³ . Anaerobic CSF culture grew the same Gram-positive rods. The fever went away after 2d of ampicillin (3 wk course, <i>i.v.</i>). CSF cultures on d 15 and were sterile. On d 38, the fever recurred and CSF was again positive for the same organism. Intravenous ampicillin followed with aqueous penicillin G was given for 26 d. On the 64 th d, intravenous chloramphenicol was initiated.	<p>The patient eventually recovered and repeated cranial computerized tomography scans and craniospinal magnetic resonance imaging were normal prior to discharge.</p> <p>CSF isolates were submitted to the Tokyo University of Agriculture and the Yakult Central Institute for Microbiological Research, and were identified as containing <i>B. breve</i>. The organism was catalase-negative, nonmotile, obligate anaerobic, Gram-positive, a non-spore-forming rod, whose major metabolites were acetic and lactic acid. DNA-DNA homology was 73% with the <i>B. breve</i> ATCC 15700 type strain. Anaerobic stool culture from the patient was positive for <i>B. breve</i>. The organism was not found in anaerobic stool and vaginal cultures from the mother.</p>	Follow-up examination at 11 mo of age indicated normal growth and development and no neurologic abnormalities.

000092

b. *Studies in Children*

No studies have evaluated the effects of *B. breve* M-16V in children. However, two studies have administered *B. breve* alone (Tojo et al., 1987; Wada et al., 2010) and two administered *B. breve* in the presence of at least one other bacterial species (Kanamori et al., 2004; Hatakka et al., 2007) (Table 15).

i. *Studies of B. breve*

Tojo and co-workers (1987) enrolled 133 patients aged 6-15 yr old who had *Campylobacter jejuni*-positive diarrhea and treated them with either 1) erythromycin for 7 d, plus antidiarrheal medication ($n = 36$), 2) 3×10^9 cfu *B. breve* BBG-01 per day for 7 d, plus antidiarrheal medication ($n = 60$), or 3) antidiarrheal medication alone ($n = 37$) until symptoms disappeared. The cumulative percentage of culture-positive patients was significantly reduced in the *B. breve* group, versus control ($P < 0.01$). *B. breve* did not have an effect on the duration of diarrhea. The authors made no mention of adverse effects.

Wada and co-workers (2010) enrolled 42 patients having malignancies who were admitted for chemotherapy and randomized them to receive either 1×10^9 cfu *B. breve* BBG-01 ($n = 19$) or cornstarch and hydroxypropyl cellulose ($n = 23$) for 2 wk prior to chemotherapy and up to 6 wk afterward or until the white blood cell count reached $> 1000/\mu\text{l}$ and the patient was discharged. Two patients were excluded from the final analysis due to having an underlying immunodeficiency (treatment group) and inadvertent exposure to *B. breve* BBG-01 (control group). *B. breve* BBG-01 did not negatively impact white blood cell and natural killer (NK) cell counts. Patients in the treatment group experienced fewer episodes and days ($p = 0.02$ for both) of fever and fewer days of parenteral antibiotic therapy ($p = 0.04$) versus the control group. *B. breve* was detected in fecal samples from the treatment group at 2, 3, 5 and 6 wk, versus baseline levels ($p < 0.01$). Total fecal short chain fatty acid content was significantly increased in the treatment group at 4 wk, versus control ($p < 0.05$). The authors state that no problems related to the study product or placebo were observed.

ii. *Studies of B. breve* in Combination with Other Probiotics

Hatakka and co-workers (2007) administered either placebo ($n = 154$) or $8-9 \times 10^9$ cfu each of *L. rhamnosus* G and LC705, *B. breve* 99 and *P. freudenreichii* ssp. *shermanii* JS ($n = 155$) per day to 309 children aged 10 mo-6 yr who had at least 4 episodes of acute otitis media (AOM) during the preceding 12 mo or ≥ 3 episodes during the preceding 6 mo. The intervention continued daily for 6 mo. The number of drop-outs was equal in both groups, one subject in the treatment group dropped out due to adverse effects that were not specified by the authors and

others dropped out because of sickness (4 in treatment group/3 in control group), non-compliance (5/8), personal reasons (5/0), tympanostomy (0/2) and unknown (5/7). The occurrence of upper respiratory infections was significantly less in the treatment group than control group ($p = 0.046$) and, although the carrier rate of the acute otitis media pathogen *Moraxella catarrhalis* in the nasopharynx was significantly increased in the treatment group, the increases in all children combined ($p = 0.028$) and the treatment group ages 1-2 yr old ($p = 0.004$) could not be attributed to specifically *B. breve* because a mixture of bacteria was used.

Kanamori and co-workers (2004) administered a total of 3×10^9 total cfu of *B. breve* Yakult and *L. casei Shirota*, plus 1 g galacto-oligosaccharides (Oligomate, Yakult Honsya, Japan) per day to seven patients having short bowels due to surgical resection (2 yr 6 mo-24 yr 8 mo in age). Bacterial preparations were given orally or via a nasogastric tube for 15-55 mo. The facultative anaerobic bacteria to aerobic bacteria ratio decreased from 46.9% to 5.73% by the end of the study. The short chain fatty acid content of wet feces was significantly increased over the study period (27.8 to 65.1 $\mu\text{mol/g}$ wet feces; $P < 0.05$) as well. Body weight gain was improved after the intervention in all but one patient (statistics not provided) who had a very short small bowel and required intravenous hyperalimentation and an elemental liquid diet.

iii. Summary of Results from Studies in Children

When administered as a single strain in children having infectious diarrhea or malignancies requiring chemotherapy, *B. breve* BBG-01 was not reported as having any adverse effects at doses of up to 3×10^9 cfu/d. When administered in conjunction with 1 g galacto-oligosaccharides and *L. casei Shirota*, *B. breve* Yakult was well-tolerated at a dose of 3×10^9 total cfu in patients having short bowels due to surgical resection. When administered as part of a probiotic mixture containing two or more organisms, *B. breve* Yakult did not increase the incidence of enterocolitis (total dose of organisms = 3×10^9 cfu/d) versus baseline incidents, whereas *B. breve* 99, in the presence of three other organisms ($8-9 \times 10^9$ cfu per strain, per capsule), may have contributed to a higher carriage rate of *Moraxella catarrhalis* in the nasopharynx of children aged 1-2 yr. Reasons for this latter observation are unknown and may be limited to the particular strain.

B. breve was shown to be safe and well tolerated in children ingesting levels up to 3×10^9 cfu/d (Tojo, et al. 1987; Kanamori, et al. 2004; Wada, et al., 2010). Importantly, because *B. breve* M-16V has been administered to infants at levels as high as 1.5×10^{10} cfu/d and to adults at levels as high as 2.0×10^{10} cfu/day, intake levels up to 1.5×10^{10} cfu of *B. breve* M-16V/d are expected to be safe in children.

000094

Table 15. Human Clinical Studies of *Bifidobacterium breve*– Children

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<i>B. breve</i>				
Tojo, et al. 1987 Highest dose tested: 3 × 10⁹ cfu/d <i>B. breve</i> BBG-01 Longest exposure: 7 d Number participants receiving <i>B. breve</i>: 60	Objective: To study the effect of <i>B. breve</i> in patients with campylobacter enteritis. Study type: Controlled Test Article: <i>B. breve</i> was from Yakult Institute (BBG-01)	One hundred thirty-three patients aged 6 mo to 15 yr who had diarrhea with culture positive for <i>Campylobacter jejuni</i> alone were enrolled in the study. Subjects received either 1) erythromycin for 7 d, plus antidiarrheal medication (<i>n</i> =36), 2) <i>B. breve</i> (BBG-01, 3 × 10 ⁹ cfu/d in three divided doses) plus antidiarrheal medication until <i>C. jejuni</i> was eradicated from the stool specimens (<i>n</i> =60) or 3) antidiarrheal medication alone until symptoms disappeared (<i>n</i> =37).	The cumulative percentage of patients with diarrhea after treatment was not significantly different among the three groups. The cumulative percentage of culture-positive patients was significantly reduced in the <i>B. breve</i> group versus control (<i>P</i> <0.01). <i>B. breve</i> did not have an effect on the duration of diarrhea.	The authors made no mention of adverse effects.
Wada, et al. 2010 Highest dose tested: 10⁹ cfu/d <i>B. breve</i> strain Yakult Longest exposure: 8 wk (6 wk on chemotherapy) Number participants receiving <i>B. breve</i>: 19	Objective: To evaluate the effects of enteral administration of <i>B. breve</i> strain Yakult in cancer patients on chemotherapy. Study type: Placebo-controlled, randomized Test Article: <i>B. breve</i> strain Yakult (BBG-01) was used	Forty-two patients having malignancies who were admitted for chemotherapy were enrolled. Exclusion criteria included congenital immunodeficiency and oral intake of probiotics during 2 wk prior to the study. Patients were randomly assigned to receive either <i>B. breve</i> strain Yakult (BBG-01) (10 ⁹ cfu/g) (<i>n</i> =19) once daily or placebo containing cornstarch and hydroxypropyl cellulose (<i>n</i> =23) for 2 wk prior to chemotherapy and continued for 6 wk or until the white blood cell count reached >1000/μl and the patient was discharged.	Two patients were excluded from the final analysis due to underlying immunodeficiency (treatment group) and BBG-01 administration during the intervention period (placebo group). Patients in the probiotic group has fewer episodes (<i>p</i> = 0.02) and days (<i>p</i> =0.02) of fever, and fewer days of parenteral antibiotic therapy (<i>p</i> =0.04) versus the placebo group. White blood cell and NK cell counts were not affected by the treatment. <i>B. breve</i> strain Yakult increased in the fecal microbiota in the treatment group at 2, 3, 5 and 6 wk, versus baseline levels (<i>p</i> <0.01). <i>Enterococcus</i> counts were decreased at 2 wk in the treatment group, versus placebo (<i>p</i> <0.05), but not at 3 wk. However, baseline <i>Enterococcus</i> counts	The authors state that no patients experienced any problems related to the study product or placebo.

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Table 15. Human Clinical Studies of *Bifidobacterium breve*– Children

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
			were higher in the treatment group compared with the placebo group. Total fecal short chain fatty acid content was significantly increased in the treatment group at 4 wk, versus placebo ($p<0.05$).	
<i>B. breve</i> Administered in Combination With Other Probiotics				
Hatakka, et al. 2007 Highest dose tested: $8-9 \times 10^9$ cfu per strain/capsule (Capsules contained <i>Lactobacillus rhamnosus</i> GG and 705, <i>B. breve</i> 99, plus <i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> JS/d Longest exposure: 6 mo Number participants receiving <i>B. breve</i>: 155	Objective: To examine whether probiotics could reduce the occurrence or duration of acute otitis media or the nasopharyngeal carriage of otitis pathogens in otitis-prone children. Study type: Double-blind, placebo-controlled, randomized Test Article: <i>B. breve</i> 99, <i>L. rhamnosus</i> GG and LC705, and <i>P. freudenreichii</i> JS were from Valio Ltd, Helsinki, Finland	Three hundred and nine children aged 10 mo to 6 yr who had at least 4 episodes of acute otitis media (AOM) during the preceding 12 mo or at least 3 episodes during the preceding 6 mo were enrolled in the study and randomized to receive one capsule of either probiotic bacteria (<i>L. rhamnosus</i> GG and 705, <i>B. breve</i> 99, plus <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS; $8-9 \times 10^9$ cfu per strain/capsule) ($n=155$) or placebo ($n=154$) per day for 6 mo.	The occurrence of ≥ 4 recurrent upper respiratory infections in the probiotic group was significantly reduced versus the placebo group ($p=0.046$). Carriage of the acute otitis media pathogen <i>Moraxella catarrhalis</i> in the nasopharynx was significantly increased in the probiotic group versus the placebo group ($p=0.028$), especially for children 1-2 yr old ($p=0.004$). The authors suggest that there may have been antagonistic effects of the four bacterial strains in combination.	The number of drop-outs was equal in both groups. Only one subject dropped out of the probiotic group due to adverse effects, but details were not provided by the study authors. Other reasons for drop-outs included: sickness (4 in probiotics group, 3 in placebo group), non-compliance (5/8), personal reasons (5/0), tympanostomy (0/2) and unknown reasons (5/7).

Table 15. Human Clinical Studies of *Bifidobacterium breve*– Children

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Kanamori, et al. 2004</p> <p>Highest dose tested: 3×10^9/d <i>B. Yakult</i> and <i>Lactobacillus casei</i> Shirota</p> <p>Exposure range: 15-55 mo</p> <p>Number participants receiving <i>B. breve</i>: 7</p>	<p>Objective: To evaluate the effect of synbiotics in short bowel patients with refractory enterocolitis.</p> <p>Study type: Uncontrolled, case studies for intervention</p> <p>Test Article: <i>B. breve</i> Yakult and <i>L. casei</i> Shirota were provided by Yakult Honsya, Japan (Biolactis Powder®)</p>	<p>Seven patients having short bowels due to surgical resection and ranging in age from 2 yr 6 mo to 24 yr 8 mo were administered <i>B. breve</i> Yakult and <i>L. casei</i> Shirota ($>10^9$ bacteria/g) plus 1 g galacto-oligosaccharide (Oligomate, Yakult Honsya, Japan) three times daily (3×10^9 bacteria total) orally or via nasogastric tube for various durations of treatment (15-55 mo). All patients suffered from repetitive enterocolitis and other general infections.</p>	<p>The average ratio of facultative anaerobic bacteria to anaerobic bacteria was decreased from 46.9% to 5.73% by the end of therapy. There was a significant increase in the average short chain fatty acid content of wet feces (27.8 to 65.09 $\mu\text{mol/g}$ wet feces; $P < 0.05$) by the end of treatment as well. Fecal levels of bifidobacteria and lactobacilli increased after synbiotic therapy (no statistics). The synbiotic therapy could not completely prevent enterocolitis.</p>	<p>Body weight gain was improved after synbiotic therapy in all but one patient. This patient had a very short small bowel and required intravenous hyperalimentation and an elemental liquid diet. Synbiotic therapy was administered for over 1 yr in all patients.</p>

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c. *Studies of B. breve in Adults*

i. *Studies of B. breve M-16V*

Two studies have evaluated the effects administering 2×10^{10} cfu *B. breve* M-16V/d to a total of 30 adults over the course of 8 weeks (Table 16) (Yoshida et al., 2010; van de Pol et al., 2011). Yoshida and co-workers (2010) enrolled 24 adults with atopic dermatitis (16F/8M; 30.2 ± 9.6 yr) and randomized them to receive either placebo ($n = 8$) or one capsule containing 1×10^{10} cfu (total dose = 2×10^{10} cfu/d) *B. breve* YY (same as *B. breve* M-16V, according to Morinaga; $n = 16$) for 8 wk. Significant favorable impact was reported on each of the following: the objective SCORAD (severity scoring of atopic dermatitis) score ($P = 0.034$; decreased), intensity criteria of SCORAD ($P = 0.018$; decreased), *Bifidobacterium* percentage of fecal microbiota ($P = 0.031$; increased), Skindex 29-J (Japanese version of a quality-of-life scoring questionnaire) ($P = 0.019$; decreased), emotions ($P = 0.030$; decreased), symptoms ($P = 0.016$; decreased). These changes were not observed in the placebo group; however, it was noted that the baseline objective and total SCORAD values were significantly higher in the probiotics group, vs. the placebo group ($P = 0.016$ and 0.027 , respectively). Also, there were six patients in the treatment group who had severe atopic dermatitis at baseline, while zero incidents were reported in the placebo group. During the study period, there was no exacerbation of allergic symptoms or adverse reactions in the form of digestive symptoms caused by the *B. breve* YY (*B. breve* M-16V) preparation.

Van de Pol et al. (2011) enrolled twenty-nine allergic adult patients with intermittent to mild persistent asthma that were capable of discontinuing short-acting β_2 -adrenoreceptor agonists for ≥ 12 hr prior to each visit and long-acting β_2 -agonists, oral antihistamines, and inhaled corticosteroids for 4 wk prior to and during the study. Blood and sputum samples were taken and bronchial hyperresponsiveness was determined on d 1. On d 2, patients were challenged with dust mite allergen, followed by blood collections at 1, 6, and 24 h. Induced sputum samples were collected at 6 and 24 h. The bronchial hyperresponsiveness test was repeated at 24 h post-challenge. After testing, subjects were randomized to receive a food supplement with ($n = 14$) or without ($n = 15$) synbiotics twice daily for 4 wk. The total dose of *B. breve* M-16V received was 2×10^{10} cfu/d. The placebo contained maltodextrin only. The synbiotics contained 1×10^{10} cfu *B. breve* M-16V plus short-chain galacto-oligosaccharides (scGOS):long-chain fructo-oligosaccharides (lcFOS) (7.2:0.8 g). Subjects tracked and recorded their peak expiratory flow rate twice/d and kept a symptom diary that tracked disease-specific and other endpoints. Inflammatory parameters did not differ between groups before and after synbiotic treatment (no

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statistics). Peak expiratory flow was increased significantly in the synbiotic group for both morning and evening measurements, at 3 and 4 wk, vs. the placebo treatment ($P < 0.05$). No significant differences in lung function were observed between the two groups during and after treatment. No long-term adverse effects were observed, according to the study authors. All blood safety parameters (total and differential leukocyte counts, IgE, interleukin 5, haemoglobin, ALAT, AST, urea, creatine and albumin levels) were normal before and after the intervention (data not shown). Two subjects dropped out of the synbiotics for personal reasons; one person in the control group was non-compliant due to intestinal side-effects. Mild gastrointestinal distress was most frequent patient complaint (3/14 in treatment group, 21.4%; 7/15 in placebo group, 46.7%).

ii. Studies of *B. breve*

Three studies administered *B. breve* to a total of 39 adults at levels ranging from 2×10^9 cfu/d to 8×10^{11} cfu/d over the course of 2 wk to 1 year (Table 16)(De Preter et al., 2008; Ishikawa et al., 2011; Shimakawa et al., 2003).

Shimakawa and co-workers (2003) evaluated the technical parameters of *B. breve* Yakult YIT 4065 for use in soymilk as a probiotic food, and included fecal recovery as an endpoint. An oral feeding study was carried out in 15 healthy males who were blindly and randomly assigned to receive either 500 mL fermented soymilk (12 h cultivation with 1.6×10^9 cfu *B. breve* Yakult YIT 4065/mL; total ingested dose of *B. breve* Yakult YIT 4065 = 8×10^{11} cfu) ($n = 8$; mean age 43.4 ± 8.6 yr) or unfermented soymilk ($n = 7$; mean age 46.6 ± 5.1 yr) daily for 2 wk. Fecal recovery of *B. breve* Yakult 4065 and total *Bifidobacterium* counts were significantly increased in the treatment group ($p < 0.05$ for both), versus control. The authors made no mention of adverse effects.

Ishikawa and co-workers (2011) enrolled 41 patients having mild to moderate ulcerative colitis and assigned them to receive either no intervention ($n = 20$; 47.4 ± 12.0 yr) or 1×10^9 cfu *B. breve* Yakult three times per day (total dose = 3×10^9 cfu/d), plus 5.5 g of galacto-oligosaccharides ($n = 21$; 43.6 ± 13.2 yr) for 1 yr. After treatment, colitis severity was determined by endoscopy. The mean endoscopic score of patients receiving treatment for 1 yr was significantly decreased vs. the control group ($p < 0.05$). In a subset of patients having active ulcerative colitis, it was found that the treatment group had significantly lower ratios of myeloperoxidase (MPO):alkaline phosphatase (ALP) at 1 yr, vs. baseline levels ($p < 0.05$); this finding was not observed in the control patients having active colitis. None of the patients in the

treatment group were hospitalized during the study. In the control group, one patient underwent a total colectomy procedure and another moved to a different hospital due to disease exaggeration.

De Preter and co-workers (2008) recruited 53 healthy volunteers (25F/28M; 19-26 yr) and assigned them to one of five treatment groups. One group received two doses of 1×10^9 cfu *B. breve* Yakult (total dose = 2×10^9 cfu) ($n = 10$), in addition to two doses of 10 g maltodextrin. The remaining groups received other strains of bacteria with either lactulose or combinations of lactulose and oligofructose-enriched inulin. Treatment with a live bacterium lasted for one 4 wk period. The ten subjects receiving *B. breve* Yakult had significantly increased levels of fecal β -glucosidase during the treatment period, vs. baseline levels ($P = 0.015$), which returned to baseline levels during the 2-week washout period. In the *B. breve* treatment group, no effects were observed on fecal dry weight or output, versus baseline levels. The authors made no mention of adverse effects.

iii. Studies of *B. breve* in Combination with Other Probiotics

Thirteen studies administered *B. breve* in the presence of one or more additional bacterial strains (Table 16)(Brigidi et al., 2001; Brigidi et al., 2003; Cha et al., 2010; Chang et al., 2011; Del Piano et al., 2010; Eguchi et al., 2011; Ishikawa et al., 2003; Kajander et al., 2005; Kajander and Korpela, 2006 (same study as reported in Kajander 2005); Kekkonen et al., 2011; Myllyluoma et al., 2005; Myllyluoma et al., 2007 (same study as Myllyluoma, et al. 2005 but were reported); Saggiaro, 2004; Shimizu et al., 2009; Taheri et al., 2011). Only five of these studies provided a *B. breve* intake level (Kajander, et al. 2005; Myllyluoma, et al. 2005; Shimizu, et al. 2009; Del Piano, et al. 2010; Kekkonen, et al. 2011), which ranged from 3×10^8 cfu/d for 40 d (Shimizu, et al. 2009) to 5×10^9 cfu/d for 21 d (Del Piano, et al. 2010). The remaining studies did not provide enough information to allow one to calculate the intake level *B. breve*. However, in these remaining studies, durations of exposure ranged from 7 d to 1 yr (Myllyluoma, et al. 2005; Myllyluoma, et al. 2007 (same study as Myllyluoma, et al. 2005 but additional endpoints are reported); Ishikawa, et al. 2002).

Of the abovementioned 13 studies, four reports were retrieved in which *B. breve* was administered in combination with one or more additional bacterial species or strains in healthy subjects (Table 16) (Brigidi, et al. 2003 (studied both healthy and health-compromised subjects); Del Piano, et al. 2010; Chang, et al. 2011; Kekkonen, et al. 2011). Brigidi and co-workers (2003) administered 6 g of VSL-3 (containing 5.58×10^{11} cfu of a combination of *B. longum*, *B. infantis*, and *B. breve*, plus multiple other species, including *S. thermophilus* and various *Lactobacillus* spp.) to five healthy (age not specified) subjects daily for 10 d. The *B. breve* fecal

concentration remained stable for 6 d after cessation of intake and the total fecal *Bifidobacterium* titer returned to baseline levels by 6 d after cessation of intake. The authors made no mention of adverse effects. Del Piano and co-workers (2010) recruited 44 healthy volunteers (23F/21M; 33-61 yr) who were randomized to receive either 5×10^9 cfu each of *Lactobacillus plantarum* LP01 and *B. breve* BR03 (non-microencapsulated) plus 2.40 g potato maltodextrin ($n = 21$) or 1×10^9 cfu of each strain (microencapsulated) plus 2.48 g potato maltodextrin ($n = 23$) for 21d, followed by a 3 wk washout period. Then the groups crossed over to the alternate treatment for an additional 21 d. Fecal counts of both lactobacilli and bifidobacteria were significantly increased in both treatment groups, versus baseline, at d10 and d 21 ($P < 0.0001$ and < 0.003 per group, respectively, for all time points). No adverse events were reported by the authors. One dropout was reported for the second group during the second treatment period (reason not provided). Chang and co-workers (2011) recruited 20-65 yr olds and randomized them to receive either a control yogurt ($n = 48$; 33F/15M) or a functional yogurt containing *B. breve* (CBG-C2) (dose not stated) plus numerous other ingredients ($n = 53$; 37F/16M). Two bottles of each yogurt were provided per day for 8 wk. It should be noted that the control yogurt contained *S. thermophilus*, *L. acidophilus*, and *B. infantis*. At wk 8, the treatment group experienced a significant decrease in body weight ($P = 0.006$), body mass index ($P = 0.006$) and LDL-cholesterol ($P = 0.044$), versus the control group. However, these changes cannot be ascribed to the presence of *B. breve* as six additional natural product extracts and functional substances were also present in the test yogurt. There were no significant changes in waist circumference, systolic and diastolic blood pressure, fasting blood glucose, HbA1c, total cholesterol, HDL, or triglycerides between the two groups or within the groups (vs. baseline levels). The authors made no mention of adverse effects. Kekkonen and co-workers (2011) recruited 18 healthy Finnish men (30-60 yr) for an intervention-only study that began with a 3 wk run-in period followed by three 2 wk subsequent interventions and a 2 wk follow-up period. The first 2 wk intervention involved ingestion of 65 mL/d of a juice that delivered a total of 3.7×10^8 cfu of *B. breve* Bb99, 6.5×10^9 cfu *P. freudenreichii* ssp. *shermanii* JS, 3.8×10^9 cfu *L. rhamnosus* GG, and 8.5×10^9 cfu *L. rhamnosus* per day. The authors made no mention of adverse effects and all participants completed the study. One participant lost 1.5 kg weight during the study (reason not stated) and one participant reported increased intake of fat and decreased intake of alcohol during the study.

Ten studies were retrieved in which *B. breve* was administered in combination with one or more additional bacterial species or strains in health-compromised patients (Table 16) (Brigidi, et al. 2001; Ishikawa, et al. 2002; Brigidi, et al. 2003 (studied both healthy and health-compromised subjects); Saggiaro 2004; Kajander, et al. 2005; Myllyluoma, et al. 2005; Kajander and Korpela 2006 (same study as reported in Kajander, et al. 2005); Myllyluoma, et al. 2007

(same study as Myllyluoma, et al. 2005 but additional endpoints are were reported); Shimizu, et al. 2009; Cha, et al. 2010; Eguchi, et al. 2011; Taheri, et al. 2011). Eight of these studies administered multiple bacterial strains in patients having gastrointestinal-related symptoms (Brigidi, et al. 2001; Ishikawa, et al. 2002; Brigidi, et al. 2003; Saggiro 2004; Kajander, et al. 2005; Myllyluoma, et al. 2005; Kajander and Korpela 2006 (same study as reported in Kajander, et al. 2005); Myllyluoma, et al. 2007 (same study as Myllyluoma, et al. 2005 but additional endpoints were reported); Cha, et al. 2010; Taheri, et al. 2011).

Four studies enrolled patients having irritable bowel syndrome (IBS) (Table 16) (Brigidi, et al. 2001; Saggiro 2004; Kajander, et al. 2005; Kajander and Korpela 2006 (same study as reported in Kajander, et al. 2005); Cha, et al. 2010). Brigidi and co-workers (2001) enrolled ten patients (24-58 yr) having diarrhea-prominent IBS or functional diarrhea according to the Rome Criteria and administered 3 g of VSL#3, a multi-species supplement containing 9.3×10^{10} cfu/g of *B. longum* Y10, *B. infantis* Y1, and *B. breve* Y8 (total of 2.79×10^{11} cfu *Bifidobacterium* spp.), per day for 20 d. VSL#3 also contained various *Lactobacillus* spp. plus *Streptococcus salivarius*. It was not possible to calculate the amount of the *B. breve* Y8 portion. Nine patients reported clinical improvement, according to the study authors; there was no mention of adverse effects and symptoms relapsed in 4/8 patients interviewed at 1 mo post-study. Saggiro (2004) enrolled 70 patients (26-64 yr; 39F/31M) with IBS according to Rome II criteria and randomized them to receive *B. breve* BR0 and *Lactobacillus plantarum* LP01 (5×10^9 cfu/mL each) ($n = 24$), *L. plantarum* LP01 plus *L. acidophilus* LA01 (5×10^9 cfu/mL each) ($n = 26$) or placebo powder containing starch ($n = 20$) twice daily for 4 wk. Note that the mL administered per day were not stated in the study report; therefore, the actual dose of the *B. breve* BR0 portion cannot be calculated. No statistics were provided, but the author stated that overall pain and symptom scores decreased in the group receiving *B. breve* BR0 at d 14 and d 28, versus baseline levels. The author made no mention of adverse effects. Kajander and co-workers (2005) enrolled 123 patients with IBS who fulfilled the Rome I or II criteria and administered either a mixture of *B. breve* Bb99 plus various *Lactobacillus* spp. and *P. freudenreichii* ssp. *shermanii* JS (total of $8-9 \times 10^9$ cfu/d; equal amounts per strain; $2-2.25 \times 10^9$ cfu/d of *B. breve* Bb99) ($n = 52$) or placebo ($n = 51$) daily for 6 mo. Patients receiving treatment experienced a significant decrease in total symptom score (abdominal pain + distension + flatulence + borborygmi) at 6 mo, versus the placebo group ($P = 0.015$). Seventeen patients withdrew from the study (8 treatment, 9 placebo). Reasons for withdrawal included illness or hospitalization for causes other than IBS (1 treatment, 3 placebo); increased GI symptoms (3 treatment, 1 placebo); desire to use other probiotic products during antibiotic treatment for other causes than IBS (2 treatment); pregnancy (2 placebo); non-compliance (1 placebo) and other reasons (2 treatment, 2 placebo). Cha and co-

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workers (2010) enrolled 47 patients having diarrhea-dominant IBS according to Rome III criteria and randomized them to receive either placebo or 7×10^{11} cfu total of *B. breve*, *B. lactis*, *B. longum*, three *Lactobacillus* spp. plus *S. thermophilus* daily for 8 wk after a 1 wk run-in period. The dose of the *B. breve* portion was not calculable. The abstract report did not state how many subjects were assigned to each group. For all weeks the proportions of adequate relief that were reported was higher in the treatment group versus the placebo group ($p < 0.05$) and patients reporting “yes” to adequate relief on 5 wk during the trial was significantly higher in the treatment group, versus placebo ($p < 0.01$). There was no difference in fecal bacterial composition observed between the two groups. The authors made no mention of adverse effects.

Four studies enrolled patients having gastrointestinal issues other than IBS, such as ulcerative colitis, inflammatory bowel disease (IBD), patients infected with *Helicobacter pylori*, and microscopic colitis (Table 16) (Ishikawa, et al. 2002; Brigidi, et al. 2003; Myllyluoma, et al. 2005; Myllyluoma, et al. 2007 (same study as Myllyluoma, et al. 2005 but additional endpoints were reported); Taheri, et al. 2011). Ishikawa and co-workers (2002) enrolled 21 patients who were diagnosed with ulcerative colitis at least 1 yr previously and assigned them to receive either no treatment ($n = 10$) or a total of 10×10^9 cfu of *B. breve*, *B. bifidum*, and *L. acidophilus* YIT 0168 as fermented milk ($n = 11$) daily for 1 yr. The dose of the *B. breve* portion was not calculable. Subjects in all groups were allowed to continue standard medical treatment for ulcerative colitis as usual on clinical grounds. Fewer subjects had symptom exacerbations in the treatment group ($p = 0.0075$) and fewer subjects had 3 or more exacerbations overall ($p = 0.009$), versus the control group. The cumulative exacerbation rate was significantly reduced in the treatment group versus control ($p = 0.0184$). Serum total protein and albumin levels were significantly increased after treatment, versus baseline levels ($p = 0.02$ and 0.03 , respectively). During the 2nd month of treatment, one subject in the treatment group developed a “coryza-like illness” with abdominal pains. Treatment was discontinued for 2 wk, and then resumed following treatment of the illness. No further abdominal pain was experienced by the subject; therefore, this symptom was thought to not be related to the treatment. The authors mention that no adverse effects which might have been related to treatment were observed in any other subjects. Brigidi and co-workers (2003) administered 6 g of VSL#3 (total of 1.8×10^{12} cfu bacteria; of which 5.58×10^{11} cfu comprised *B. breve*, *B. longum*, and *B. infantis*, plus four *Lactobacillus* ssp. and *S. thermophilus*) to ten patients having IBD daily for 10 d. It was not possible to calculate the amount of *B. breve* alone. *B. breve* Y8 was observed via polymerase chain reaction (PCR) carried out on fecal samples from these patients at 2 mo after the study, indicating that the bacterium persists in the feces at least this long in IBD patients. The authors made no mention of adverse effects. Myllyluoma and co-workers (2005 and 2007) enrolled 52 patients testing

positive for *H. pylori* via a ^{13}C -urea breath test and serology. Of the 52 subjects, 5 did not meet eligibility criteria and 47 were randomized to receive either placebo ($n = 24$) or treatment with a multi-bacterial preparation containing *B. breve* Bb99 (DSM 13692), two strains of *L. rhamnosus*, plus *P. freudenreichii* ssp. *shermanii* JS in a milk-based drink ($n = 23$). All participants received 7 d of triple antibiotic eradication therapy. During this 7 d period, the treatment group received 9.1×10^8 cfu *B. breve* Bb99 (plus other organisms) per day, followed by 4.55×10^8 cfu *B. breve* Bb99 (plus other organisms) per day for 3 wk. The total symptom score change during the antibiotic therapy period was significantly reduced in the treatment group versus placebo ($P < 0.05$). Defecation frequency was similar between both groups. Concentrations of bacteria detected in the feces returned to baseline levels after the 6 wk follow up period. Bacterial supplementation did not reduce the effectiveness of the triple antibiotic therapy against *H. pylori*; in fact, antibiotic response rates were somewhat higher in the treatment group versus the control group (not statistically significant). There were no differences between the groups regarding the occurrence of new or aggravated symptoms. The authors made no mention of adverse effects. Taheri and co-workers (2011) enrolled 52 patients with microscopic colitis and administered them 1 mg loperamide per day in addition to either a placebo ($n = 25$) or a bacterial mixture (5×10^9 cfu of a mixture of *B. breve* Rosell-70 plus *B. longum* Rosell-175, *B. bifidum* Rosell-71, two *Lactobacillus* spp. and *L. lactis* Rosell-1058) ($n = 27$) per day for 4 wk. The dose of *B. breve* as cfu/d was not provided. Forty-six subjects had lymphocytic colitis and six had collagenous colitis. In the patients presenting with lymphocytic colitis, abdominal pain and frequency of defecation were significantly reduced in the treatment group versus placebo ($p < 0.001$). Serious complications were not observed in either group, according to the authors. One patient in the treatment group dropped out due to intensified diarrhea.

Two studies were carried out with other health-compromised subjects (Table 16) (Shimizu, et al. 2009; Eguchi, et al. 2011). Shimizu and co-workers administered 3×10^8 cfu *B. breve* Yakult plus 3×10^8 cfu *L. casei* Shirota and 10 g galacto-oligosaccharides per day to 29 patients having severe systemic inflammatory response syndrome (SIRS) for 40 d. Twenty-six previously severe SIRS patients served as controls and were not treated. Enteral nutrition was initiated as soon as possible in both groups. Infections were initially treated based on clinical presentation and therapy was adjusted according to results from bacterial isolate resistance testing. Incidences of enteritis, pneumonia, and bacteremia were significantly decreased in the treatment group versus control ($P < 0.05$). Septic mortality was also reduced but did not reach statistical significance. The authors state that all patients in the treatment group tolerated the treatment well and that there were no adverse events in any patient. Eguchi and co-workers (2011) enrolled 50 liver transplant recipients and randomly assigned them to receive either no

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therapy ($n = 25$; 9F/16M; 25-68 yr old) or *B. breve* Yakult (45 mg), *L. casei* Shirota (60 mg), plus 45 g galacto-oligosaccharides daily beginning 2 d preoperatively and continuing for 2 wk (either via tube jejunostomy or orally) after elective living donor liver transplantation. The dose of *B. breve* as cfu/d was not provided. There was no significant change in the pattern of fecal bacterial species between the two groups. Infectious complications were significantly reduced in the treatment group versus the control ($P < 0.05$). The authors stated that some infectious complications occurred after the treatment was discontinued. There were no significant differences between groups regarding other complications after transplantation, the intensive care unit period, hospitalized period, and mortality rate. There was no difference between the groups in the rejection rate, even though there were more blood group-incompatible transplant patients in the synbiotic group than in the control group.

iv. Summary of Results from Adult Studies

Two studies administered Morinaga's *B. breve* M-16V at 2×10^{10} cfu/d (Van de Pol, et al. 2010; Yoshida, et al. 2010), with mild gastrointestinal complaints being the only reported adverse effects in adult subjects having allergic asthma. *B. breve* has been administered in adults as a single species at doses ranging from 2×10^9 cfu/d (4 wk; De Preter, et al. 2008) to 8×10^{11} cfu/d (2 wk; Shimakawa, et al. 2003) and for durations ranging from 2 wk (1.6×10^9 cfu/mL in soymilk, total dose of 8×10^{11} cfu/d; Shimakawa, et al. 2003) up to 1 yr (3×10^9 cfu/d; Ishikawa, et al. 2011). When administered as part of a bacterial mixture, *B. breve* was given at doses ranging from 3×10^8 cfu/d (40 d; Shimizu, et al. 2009) to 5×10^9 cfu/d (7 d; Del Piano, et al. 2010) and for durations ranging from 7 d (Myllyluoma, et al. 2005 and 2007) to 1 yr (Ishikawa, et al. 2002).

B. breve M-16V is safe and well tolerated in adults ingesting levels up to 2×10^{10} cfu/d (Van de Pol, et al. 2010; Yoshida, et al. 2010), and *B. breve* is safe and well tolerated at up to 8×10^{11} cfu/d (Shimakawa, et al. 2003).

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcomes	Safety-Related Observations
<i>B. breve</i> M-16V				
Yoshida, et al. 2010 Highest dose tested: 2 × 10¹⁰ cfu/d <i>B. breve</i> strain YY/d Longest exposure: 8 wk Number participants receiving <i>B. breve</i> M-16V: 16	<i>Objective:</i> To evaluate the effect of <i>B. breve</i> on atopic dermatitis. <i>Study type:</i> Placebo-controlled, randomized <i>Test Article:</i> <i>B. breve</i> strain YY was from Tokiwa Pharmaceutical, Kobe, Japan < Note: Morinaga states that <i>B. breve</i> strain YY is the same as <i>B. breve</i> M-16V >	Twenty-four adult patients (8M/16W; mean age 30.2 ± 9.6 yr) diagnosed with atopic dermatitis were enrolled. The patient's pre-study treatment regimen was maintained during the study. Sixteen subjects received one capsule containing <i>B. breve</i> strain YY (1.0 × 10 ¹⁰ cfu/capsule) twice per day for 8 wk (total of 2.0 × 10 ¹⁰ cfu per day). Eight subjects received placebo capsules. Dermatitis severity was assessed using the severity scoring of atopic dermatitis (SCORAD) scoring system and quality of life was evaluated using the Japanese version of the Skindex-29 questionnaire. Fecal sampling and blood collection were also carried out. <u>Note:</u> Baseline objective and total SCORAD values were significantly higher in the probiotics group, versus the placebo group (P=0.016 and 0.027, respectively). Also, the number of patients having severe atopic dermatitis was 6 in the probiotics group and 0 in the placebo group.	The following parameters were significantly improved in the probiotics group at 8 wk, versus baseline levels: objective SCORAD (P=0.034), intensity criteria of SCORAD (improved; P=0.018), <i>Bifidobacterium</i> percent of fecal microbiota (increased; P=0.031), Skindex 29-J (Japanese version of quality-of-life scoring questionnaire; decrease; P=0.019), emotions (P=0.030), and symptoms (decreased; P=0.016). These changes were not observed at 8 wk in the placebo group.	During the study period, there was no exacerbation of allergic symptoms or adverse reactions in the form of digestive symptoms caused by the test article.

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Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Van de Pol, et al. 2011 Highest dose tested: 2×10^{10} cfu/d Longest exposure: 4 wk Number participants receiving <i>B. breve</i> M-16V: 29	<p>Objective: To determine the effect of synbiotics, galacto-oligosaccharides, and long-chain fructo-oligosaccharides on allergic responses in adults with allergic asthma.</p> <p>Study type: Randomized, double-blind, parallel</p> <p>Test Article: Probiotics contained <i>B. breve</i> M-16V (Morinaga Milk Industry Co., Ltd) plus short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (7.2:0.8 g).</p>	<p>Twenty-nine allergic adult patients with intermittent to mild persistent asthma who were able to stop short-acting β_2-adrenoreceptor agonists for ≥ 12 hr before each visit, and long-acting β_2-agonists, oral antihistamines, and inhaled corticosteroids for 4 wk prior to and during the study were enrolled. Blood and sputum samples were taken and bronchial hyperresponsiveness was determined with PC₂₀methacholine on day 1. On d 2, patients were challenged with house dust mite allergen, and blood was collected 1, 6, and 24 hr post-challenge. Induced sputum samples were collected at 6 and 24 hr post-challenge, and the PC₂₀methacholine test was repeated at 24 hr post-challenge. After the initial round of testing, subjects were randomized to receive a food supplement with (n=14) or without (n=15) synbiotics. The synbiotics contained <i>B. breve</i> M-16V (10^{10} cfu) plus scGOS/lcFOS (7.2:0.8 g) and were administered twice daily for 4 wk. The placebo consisted of maltodextrin only. During the intervention, subjects measured their peak expiratory flow twice daily and kept a symptom diary that tracked disease-specific and additional endpoints.</p>	<p>Inflammatory parameters in sputum did not appear to differ between groups before and after synbiotic treatment (no statistics). At weeks 3 and 4, changes from baseline for morning ($P < 0.05$ and < 0.01, respectively) and evening ($P < 0.01$ and < 0.05, respectively) peak expiratory flow were significantly increased in the synbiotics group, versus placebo. No significant differences in changes in lung function, such as forced expiratory volume in 1 s, changes in asthma scores, short-acting β_2-agonist usage, and PC₂₀methacholine (pre- and post-challenge) were observed during and after treatment. After synbiotics treatment, levels of serum interleukin-5 (IL-5) did not increase at 24 h post-bronchial allergen challenge in the synbiotics group, versus the control group ($P < 0.034$).</p>	<p>No long-term adverse effects were observed, according to the study authors. All blood safety parameters were normal before and after intervention (data not shown).</p> <p>Two subjects dropped out of the synbiotics group for personal reasons. One subject in the control group was non-compliant due to intestinal side-effects. Mild gastrointestinal complaints were reported most often and slightly more in the placebo group (3/14) versus treatment group (7/15).</p>

000107

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<i>B. breve</i>				
Shimakawa, et al. 2003 Highest dose tested: 8 × 10¹¹ cfu/d <i>B. breve</i> Yakult YIT 4065 Longest exposure: 2 wk Number participants receiving <i>B. breve</i>: 8	<i>Objective:</i> To evaluate <i>B. breve</i> Yakult YIT 4065 for use in soymilk as a probiotic food. <i>Study type:</i> Technical evaluation of strain plus a blinded, controlled oral feeding experiment. <i>Test Article:</i> <i>B. breve</i> Yakult YIT 4065	Various <i>in vitro</i> technical parameters were evaluated for <i>B. breve</i> Yakult YIT 4065 for use as a probiotic in fermented soymilk. Fecal survivability after oral administration was also evaluated in a clinical study. For the oral feeding study, 15 healthy males were blindly and randomly assigned to 2 groups 1 wk prior to sample administration, and were given either 500 mL fermented soymilk (12 h cultivation, 1.6 × 10 ⁹ cfu/ mL <i>B. breve</i> Yakult YIT 4065 (n=8; mean age 43.4 ± 8.6 yr) or unfermented soymilk (n=7; mean age 46.6 ± 5.1 yr) daily for 2 wk.	<i>B. breve</i> Yakult YIT 4065 reduced the pH of fermented soymilk to < 4.8 over ~24 h and survived storage at 10°C in fermented soymilk for at least 20 d. Growth of this strain was inhibited by bile but this effect was overcome by the presence of 0.2 and 0.5% soy protein (no statistics). Fecal <i>B. breve</i> Yakult and total <i>Bifidobacterium</i> counts were significantly increased in the treatment group (p<0.05 for both) versus control.	The authors made no mention of adverse effects.

000108

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Ishikawa, et al. 2011 Highest dose tested: 3 × 10 ⁹ cfu/d <i>B. breve</i> Yakult+ 5.5 g galacto-oligosaccharides Longest exposure: 1 yr Number participants receiving <i>B. breve</i>: 21	<p><i>Objective:</i> To study the effects of <i>B. breve</i> and galacto-oligosaccharides on patients with ulcerative colitis.</p> <p><i>Study type:</i> Single-center, randomized, controlled</p> <p><i>Test Article:</i> The probiotic was <i>B. breve</i> Yakult and galacto-oligosaccharides (55% as a gel) were concurrently administered.</p>	Forty-one patients with mild to moderate ulcerative colitis were assigned to one of two groups: 1) one gram of freeze-dried powder containing 10 ⁹ cfu of <i>B. breve</i> Yakult/g ×3 per day, plus 5.5 g of galacto-oligosaccharides/d (n=21; 43.6 ± 13.2 yr old) or 2) no intervention (n=20; 47.4 ± 12.0 yr old) for 1 yr. After treatment, colitis severity was estimated by endoscopy. Background medications such as salazosulfapyridine, mesalazine and steroids were continued in both groups as needed.	<p>The mean endoscopic score of patients receiving synbiotics for 1 yr was significantly decreased versus the control group (p<0.05). The lavage solution from patients receiving synbiotic treatment did not increase in its myeloperoxidase (MPO):alkaline phosphatase (ALP) levels, whereas an increase was observed in the control group at 1 yr, versus baseline levels (statistics not provided for treatment group; significant increase after treatment vs. baseline in the control group, p<0.05). The authors noted that MPO:ALP values are lower in inactive, versus active, ulcerative colitis. When a subset of patients having active ulcerative colitis was analyzed, the synbiotic therapy was found to significantly decrease their MPO:ALP levels at 1 yr, versus baseline (p<0.05). This decrease was not observed in the active colitis patients in the control group.</p> <p>Recovery of administered <i>B. breve</i> Yakult was 10^{5.75 ± 1.65} cfu/g of feces after the synbiotic treatment.</p> <p><i>Bacteroidaceae</i> and fecal pH were significantly reduced at 1 yr, versus baseline, in the treatment group (p<0.05). <i>Bifidobacterium</i> as a group were not significantly changed, but <i>B. breve</i> was detected after treatment (see above).</p>	All synbiotic treatment subjects completed the regimen and none were hospitalized during the study. In the control group, one subject underwent whole colectomy and one moved to another hospital due to exaggeration of disease.

000109

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>De Preter, et al. 2008</p> <p>Highest dose tested: 2×10^9 cfu/d <i>B. breve</i> Yakult</p> <p>Longest exposure: 4 wk</p> <p>Number participants receiving <i>B. breve</i>: 10</p>	<p><i>Objective:</i> To study the effect of various pre- and probiotics on fecal β-glucuronidase and β-glucosidase activity.</p> <p><i>Study type:</i> Randomized, cross-over</p> <p><i>Test Article:</i> <i>B. breve</i> Yakult (Yakult Honsha Co. Ltd, Tokyo, Japan) was used as a probiotic in combination with maltodextrin.</p>	<p>Fifty-three healthy volunteers (25F/28M; aged 19-26 yr) were randomly assigned to one of five treatment groups. Each group contained 10-11 subjects and received 3-4 four-week treatments, with 2 wk washout periods following each treatment period. Only one of these groups received two doses of 1×10^9 cells <i>B. breve</i> Yakult (total dose = 2×10^9) ($n=10$), in addition to two doses of 10 g maltodextrin. Treatment with active probiotic lasted only one 4 wk period.</p>	<p>Fifty participants completed the study. Of these, ten (6M/4F) were in the <i>B. breve</i> treatment group. During the treatment period, levels of β-glucosidase increased significantly ($P = 0.015$), versus baseline levels, and then returned to baseline during the washout period.</p>	<p>One male and one female subject withdrew from the study due to the need to take antibiotics (neither was in the <i>B. breve</i> group). Another female subject withdrew due to personal reasons (also not in the <i>B. breve</i> group).</p> <p>In the <i>B. breve</i> treatment group, no effects were observed on fecal dry weight or fecal output, versus baseline levels. The authors made no mention of adverse effects.</p>

000110

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<i>B. breve</i> Administered in Combination With Other Probiotics				
Brigidi, et al. 2001	<p><i>Objective:</i> To study the effect of probiotics in patients with IBS or functional diarrhea.</p> <p><i>Study type:</i> Not stated</p> <p><i>Test Article:</i> VSL#3 contains: 9.3×10^{10} cfu/g of <i>Bifidobacterium</i> (<i>B. longum</i> Y10, <i>B. infantis</i> Y1 and <i>B. breve</i> Y8/d plus other bacteria (cannot calculate dose of <i>B. breve</i> alone)</p> <p><i>Longest exposure:</i> 20 d</p> <p><i>Number participants receiving B. breve:</i> 10</p> <p>VSL#3 contains: 9.3×10^{10} cfu/g of <i>Bifidobacterium</i> (<i>B. longum</i> Y10, <i>B. infantis</i> Y1 and <i>B. breve</i> Y8), 2.7×10^9 cfu/g of <i>Lactobacillus</i> (<i>Lactobacillus acidophilus</i>, <i>Lactobacillus casei</i>, <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> and <i>Lactobacillus plantarum</i>) and 2×10^{11} cfu/g of <i>Streptococcus salivarius</i> subsp.</p>	Ten patients aged 24-58 yr who had diarrhea-prominent IBS for at least 2 yr or functional diarrhea according to the Rome Criteria were selected. A 2 wk washout period ensued followed by administration of 3 g of VSL#3/d for 20 d. Fecal samples were analyzed at d 0 and 20 d, and 10 d after ending treatment.	Nine patients reported clinical improvement, according to the study authors. Five patients considered the change in symptoms “excellent” and 3 as “good” in comparison with the initial intensity. Symptoms relapsed in 4/8 patients interviewed at 1 mo post-study. Both supplemented organisms were detected in the feces at 20 d and <i>Streptococcus thermophilus</i> populations were increased at 20 d as well, versus baseline levels (no statistics). Fecal urease levels decreased and beta-galactosidase levels increased at 20 d, relative to baseline levels (no statistics).	The authors made no mention of adverse effects.

000111

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Del Piano, et al. 2010 Highest dose tested: 5×10^9 cfu/d <i>B. breve</i> BR03 plus same amount <i>L. plantarum</i> LP01 Longest exposure: 21 d Number participants receiving <i>B. breve</i>: 44	<p><i>Objective:</i> To compare the intestinal colonization efficiency of microencapsulated versus non-microencapsulated probiotics.</p> <p><i>Study type:</i> Double-blind, randomized, cross-over</p> <p><i>Test Article:</i> The probiotic contained <i>L. plantarum</i> LP01 (LMB P-21021) and <i>B. breve</i> BR03 (DSM 16604) in either an uncoated form or microencapsulated with a gastroresistant material. Bacterial strains were from Probiotical, Novara, Italy.</p>	Forty-four healthy volunteers were enrolled in the study (21M/23F; 33-61 yr old). Subjects were randomly assigned to receive sachets containing either 1) <i>L. plantarum</i> LP01 and <i>B. breve</i> BR03 (non-microencapsulated; 5×10^9 cfu/strain/sachet; plus 2.40 g potato maltodextrin) or 2) the same bacteria, microencapsulated (1×10^9 cfu/strain/sachet; plus 2.48 g potato maltodextrin) daily for 21 d, followed by a 3 wk washout period. Subjects then crossed over to the alternate treatment for an additional 21 d. Fecal samples were collected on days 0, 10, and 21 of each treatment phase.	Fecal counts of both Lactobacilli and Bifidobacteria (separate readouts) were significantly increased in the non-microencapsulated group at d 10 and d 21, versus baseline levels ($P \leq 0.0001$ for all time points). The same effect was observed in the microencapsulated group, except that the <i>P</i> values were slightly larger ($P \leq 0.003$ for all time points). After the 21 day washout period, fecal concentrations of the two genera were similar to those recorded at baseline.	No adverse events were reported by the authors and only one dropout was recorded for group 2 during the second treatment period.

000112

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Kajander, et al. 2005 Highest dose tested: $2-2.25 \times 10^9$ cfu/d <i>B. breve</i> Bb99 plus other strains Longest exposure: 6 mo Number participants receiving <i>B. breve</i>: 52	<p><i>Objective:</i> To determine the effect of a probiotic mixture in IBS patients.</p> <p><i>Study type:</i> Randomized, double-blind, controlled</p> <p><i>Test Article:</i> The probiotic mixture contained <i>L. rhamnosus</i> GG, <i>L. rhamnosus</i> LC705, <i>B. breve</i> Bb99 and <i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> JS and was from Valio Ltd, Helsinki, Finland.</p>	One hundred three patients with IBS who fulfilled the Rome I or II criteria were enrolled. The trial was preceded by a 1 wk baseline period, after which patients received one capsule containing <i>L. rhamnosus</i> GG, <i>L. rhamnosus</i> LC705, <i>B. breve</i> Bb99 and <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS ($8-9 \times 10^9$ total cfu/d; equal amounts of each strain per capsule) ($n=52$) or a placebo capsule ($n=51$) daily for 6 mo.	Patients in the treatment group had a significant decrease in total symptom score (abdominal pain + distension + flatulence + borborygmi) during the intervention, versus the placebo group ($P=0.015$). Borborygmi and total symptom score were significantly reduced in the probiotic group versus placebo from baseline to the second half of the study ($P=0.035$ and 0.002 , respectively) and during the second half of the study ($P=0.008$ and 0.035). Abdominal pain and flatulence were only reduced from baseline to the second half of the study in the probiotic group, versus placebo ($P=0.035$ and 0.011).	Seventeen patients withdrew due to illness. Reasons included: hospitalization for other causes than IBS (1 in the probiotic group, three in the placebo group), subjective increase in symptoms (3 probiotic, 1 placebo), desire to use other probiotic products during antimicrobial treatment for other causes than IBS (2 probiotic), pregnancy (2 placebo), non-compliance (1 placebo), and other reasons (2 probiotic, 2 placebo). The authors mention that no apparent adverse effects were observed.

000113

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Kajander and Korpela 2006</p> <p>[Note: Same study as reported on in Kajander, et al. 2005]</p> <p>Highest dose tested: 2-2.25 × 10⁹ cfu/d B. breve Bb99 plus other strains</p> <p>Longest exposure: 6 mo</p> <p>Number participants receiving B. breve: 44</p>	<p><i>Objective:</i> To evaluate the clinical efficacy of a probiotic combination in IBS patients.</p> <p><i>Study type:</i> Randomized, double-blind, controlled</p> <p><i>Test Article:</i> The probiotic combination contained <i>L. rhamnosus</i> GG, <i>L. rhamnosus</i> Lc705, <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS, and <i>B. breve</i> Bb99</p>	<p>One hundred three patients with a well-established diagnosis of IBS that fulfilled the Rome criteria I and/or II were enrolled, and 86 subjects completed the study. Subjects took either a placebo capsule or one multispecies probiotic capsule (total bacteria 8-9 × 10⁹ cfu/d; equal amounts of each: <i>L. rhamnosus</i> GG, <i>L. rhamnosus</i> Lc705, <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS, and <i>B. breve</i> Bb99) for 6 mo.</p> <p><u>Note:</u> Published report did not state how many subjects were assigned to each group</p>	<p>At the end of the intervention, the total symptom score (abdominal pain + distension + flatulence + rumbling; possible range 0-112) had reduced 42% in the treatment group, versus placebo (no statistics). At 6 mo the baseline-adjusted total symptom score was 7.7 points lower in the probiotic group versus the placebo group (<i>P</i>=0.015). There were no significant differences between the groups regarding changes in bowel habits or quality of life, according to the study authors.</p>	<p>The authors made no mention of adverse effects.</p>

000114

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Myllyluoma, et al. 2005</p> <p>Highest dose tested: 9.1×10^8 cfu/d <i>B. breve</i> Bb99 plus other strains</p> <p>Longest exposure: 7 d at high dose (9.1×10^8 cfu <i>B. breve</i> Bb99), followed by 3 wk at lower dose (4.55×10^8 cfu <i>B. breve</i> Bb99)</p> <p>Number participants receiving <i>B. breve</i>: 23</p>	<p>Objective: To investigate the effect of probiotic supplementation in <i>H. pylori</i>-positive patients.</p> <p>Study type: Randomized, double-blind, controlled, pilot</p> <p>Test Article: The probiotic mixture contained <i>L. rhamnosus</i> GG + <i>L. rhamnosus</i> LC (DSM 7061) + <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS (DSM 7076) + <i>B. breve</i> Bb99 (DSM 13692) and was from Valio Ltd, Helsinki, Finland.</p>	<p>Fifty-two patients testing positive for <i>H. pylori</i> via ^{13}C-urea breath test and serology were enrolled. Five did not meet eligibility criteria and a total of 47 patients were randomized to either placebo ($n=24$) or probiotics ($n=23$). All participants received a 7 d triple antibiotic therapy (30 mg lansoprazole, 500 mg clarithromycin and 1 g amoxicillin, 2x/d), followed by 65 mL of a milk-based fruit drink containing 1×10^9 cfu/mL of <i>L. rhamnosus</i> GG + <i>L. rhamnosus</i> LC (6×10^8 cfu total lactobacilli/mL) + <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS (7×10^8 cfu/mL) + <i>B. breve</i> Bb99 (7×10^6 cfu/mL) or the same drink without probiotics twice per day during the antibiotic therapy and once per day for 3 wk afterwards.</p>	<p>The total symptom score change during eradication week (antibiotic therapy) was significantly reduced in the probiotic group versus the placebo group ($P<0.05$). No significant differences were observed in defecation frequency between the two groups. Fecal concentrations of <i>L. rhamnosus</i> GG and <i>P. freudenreichii</i> JS were significantly increased in the probiotics group versus placebo at 1 ($P<0.001$ for both) and 4 wk ($P<0.001$ for both). The concentrations of probiotic bacteria returned to baseline values after the 6 wk follow-up period. Eradication rates for <i>H. pylori</i> infection were higher in the probiotic versus the placebo group but the difference was not statistically significant ($P=0.42$).</p>	<p>Probiotic supplementation did not reduce the effectiveness of the triple antibiotic therapy against <i>H. pylori</i>; in fact, it enhanced it. There were no differences between the two groups as to the occurrence of new or aggravated symptoms.</p>

000115

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcomes	Safety-Related Observations
<p>Myllyluoma, et al. 2007</p> <p>[Note: Same study as reported in Myllyluoma, et al. 2005]</p> <p>Highest dose tested: 9.1×10^8 cfu/d <i>B. breve</i> Bb99 plus other strains</p> <p>Longest exposure: 7 d at high dose (9.1×10^8 cfu <i>B. breve</i> Bb99) 3 wk at lower dose (4.55×10^8 cfu <i>B. breve</i> Bb99)</p> <p>Number participants receiving <i>B. breve</i>: 23</p>	<p>Objective: To evaluate the effect of probiotics on intestinal microbiota in patients being treated for <i>H. pylori</i> infection.</p> <p>Study type: Randomized, double-blind, placebo-controlled</p> <p>Test Article: The probiotic contained <i>L. rhamnosus</i> GG (ATCC 53103), <i>L. rhamnosus</i> LC705 (DSM 7061), <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS (DSM 7067), and <i>B. breve</i> Bb99 (DSM 13692) (Valio Ltd., Helsinki, Finland).</p>	<p>Forty-seven <i>H. pylori</i>-positive adults (^{13}C-urea breath test and enzyme immunoassay serology) (10M/13F; mean age 57.3 yr) participated in the study. These subjects were randomized to receive either 1) probiotic therapy, 1×10^9 cfu/mL as a milk-based fruit drink (probiotic contained <i>L. rhamnosus</i> GG, <i>L. rhamnosus</i> LC705, <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS, and <i>B. breve</i> Bb99; volume not stated) plus triple antibiotic therapy (40 mg lansoprazole, 500 mg clarithromycin, and 1 g amoxicillin, 2×d) ($n=23$) or 2) a placebo (same drink, without probiotics) plus triple antibiotic therapy ($n=24$). The antibiotics were administered for 7 d. During this time the probiotic was consumed twice daily and then once daily for an additional 3 wk. A non-infected control group was also included ($n=19$; 3M/16F; mean age 44.3 yr). Fecal samples were collected for microbiota analysis.</p>	<p>The lactobacilli/enterococci populations (assessed by fluorescence <i>in situ</i> hybridization; FISH) were significantly increased in the probiotic group at d 7 and d 28, versus the placebo group ($P<0.05$ for both). Using the FISH method <i>Faecalibacterium prausnitzii</i> was also observed to be significantly decreased in the probiotics group at d 70 versus the placebo group ($P<0.01$). Total aerobes, assessed via plate count, were significantly higher in the probiotic group at d 7 and d 28, versus the placebo group ($P<0.05$). <i>Propionibacterium</i> spp. were eliminated in the probiotics group at d 7, versus the placebo group ($P<0.01$); however, the population had recovered to baseline levels by d 28 and d 70.</p>	<p>The authors made no mention of adverse effects.</p>

000116

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Kekkonen, et al. 2011 Highest dose tested: 3.7×10^8 cfu <i>B. breve</i> strain Bb99/d Longest exposure: 2 wk Number participants receiving <i>B. breve</i>: 18	<p><i>Objective:</i> To examine the effect of probiotics and synbiotics on fecal β-glucosidase activity and serum enterolactone concentrations in men.</p> <p><i>Study type:</i> Intervention-only, progressive interventions (only the probiotic stage is included in the summary here)</p> <p><i>Test Article:</i> Probiotic juice (Valio) contained <i>L. rhamnosus</i> GG (ATCC 53103; 5.9×10^{10} cfu/L), <i>L. rhamnosus</i> LC705 (DSM7061; 1.3×10^{11} cfu/L), <i>B. breve</i> Bb99 (DSM13692; 5.7×10^9 cfu/L), and <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS (DSM7067; 1×10^{11} cfu/L).</p>	Eighteen healthy Finnish men aged 30-60 yr (mean 45 yr) volunteered for the study. The study lasted for 11 wk and consisted of a 3 wk run-in period followed by three 2 wk subsequent interventions and a final 2 wk follow-up period. The first 2 wk intervention involved the ingestion of 65 mL/d of a juice containing probiotic bacteria (<i>L. rhamnosus</i> GG (5.9×10^{10} cfu/L; 3.8×10^9 cfu/d), <i>L. rhamnosus</i> LC705 (1.3×10^{11} cfu/L; 8.5×10^9 cfu/d), <i>B. breve</i> Bb99 (5.7×10^9 cfu/L; 3.7×10^8 cfu/d), and <i>P. freudenreichii</i> ssp. <i>shermanii</i> JS (1×10^{11} cfu/L; 6.5×10^9 cfu/d). Blood and fecal samples were taken and questionnaires were completed by participants.	During the probiotics intervention, fecal total propionibacteria, <i>L. rhamnosus</i> LC705 and <i>L. rhamnosus</i> LGG were significantly increased, versus run-in levels ($p < 0.01$).	The authors made no mention of adverse effects. All participants completed the study. One participant lost 1.5 kg during the study (reason not stated) and one participant reported that his intake of fat and alcohol decreased during the study.

000117

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Shimizu, et al. 2009</p> <p>Highest dose tested: 3×10^8 cfu/d <i>B. breve</i> strain Yakult plus 3×10^8 cfu <i>L. casei</i> strain Shirota</p> <p>Longest exposure: 40 d</p> <p>Number participants receiving <i>B. breve</i>: 29</p>	<p>Objective: To determine whether synbiotic therapy can improve gut flora in patients having severe systemic inflammatory response syndrome (SIRS).</p> <p>Study type: Controlled, non-randomized</p> <p>Test Article: Probiotics used were Yakult BL Seichoyaku containing 1×10^8 living <i>B. breve</i> Yakult/g and 1×10^8 living <i>L. casei</i> Shirota/g (Yakult Honsha, Tokyo, Japan). Prebiotics were galacto-oligosaccharides (Oligomate HP, Yakult Honsha).</p>	<p>Twenty-nine severe systemic inflammatory response syndrome (SIRS) patients were treated with synbiotics, which contained 1×10^8 living <i>B. breve</i> Yakult/g and 1×10^8 living <i>L. casei</i> Shirota/g (3 g of formulation/d), plus galacto-oligosaccharides (10 g/d) for 40 d. Twenty-six previously severe SIRS patients served as controls and were not treated. Enteral nutrition was initiated as soon as possible in both groups. Infections in both groups were initially treated based on clinical presentation and then according to the results of resistance testing of the isolated bacterial infection. Fourteen healthy volunteers were included for comparison. Fecal samples were collected for analysis of flora and organic acids.</p>	<p>Fecal flora counts of <i>Bifidobacterium</i> spp. increased significantly on days 1-10, 11-20, and 21-40 in the treatment group, versus control ($P < 0.05$ for all). <i>Lactobacillus</i> counts also increased in the synbiotic group, versus control, on days 11-20 and 21-40 ($P < 0.05$ for both). <i>Bacteroidaceae</i> counts increased as well, versus control, on days 21-40 ($P < 0.05$); however, the control group had lower counts in general, versus earlier time periods.</p> <p>Fecal acetic and propionic acid content was significantly increased in the synbiotic group, versus control, on days 1-10, 11-20, and 21-40 ($P < 0.05$ for all). Total fecal organic acid content was increased on days 1-10, 11-20, and 21-40 in the treatment group, versus control ($P < 0.05$ for all). Fecal pH was significantly reduced on days 21-40 in the synbiotics group, versus control ($P < 0.05$).</p> <p>The incidences of enteritis, pneumonia, and bacteremia were significantly reduced in the synbiotics group versus control ($P < 0.05$). Septic mortality was also decreased, but did not attain statistical significance.</p>	<p>The authors state that all patients in the synbiotics group tolerated the treatment well and that there were no adverse events in any patient.</p>

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Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Cha, et al. 2010 (Abstract) Highest dose tested: Mixture of <i>B. breve</i> plus other strains (dose of <i>B. breve</i> not calculable) Longest exposure: 8 wk Number participants receiving <i>B. breve</i>: less than 47	Objective: To evaluate the effects of a probiotic mixture in patients with diarrhea-dominant IBS. Study type: Placebo-controlled, double-blind, randomized Test Article: The probiotic contained 7×10^{11} cfu total of <i>S. thermophilus</i> , <i>L.</i> <i>plantarum</i> , <i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>B. lactis</i> , <i>B.</i> <i>longum</i> , and <i>B. breve</i> . (VSL#3)	Forty-seven patients having diarrhea- dominant IBS according to ROME III criteria were randomized to receive either probiotics or placebo daily for 8 wk after a 1 wk run-in period. The probiotic treatment contained 7×10^{11} cfu total of <i>S.</i> <i>thermophilus</i> , <i>L. plantarum</i> , <i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>B. lactis</i> , <i>B. longum</i> , and <i>B.</i> <i>breve</i> . The primary outcome was adequate relief for overall IBS symptoms, and secondary outcomes included individual symptoms and quality of life. There was a 2 wk follow-up period.	Patients reporting “yes” to adequate relief on half of the weeks (5 wk) during the trial was significantly higher in the treatment group, versus placebo ($p < 0.01$). However, improvements of individual symptom scores and stool parameters were not superior in the probiotics group. There was no difference in the similarities of the bacterial composition between the two groups. Therefore, the effect of the probiotics on improving overall IBS symptoms was judged to be not related to the compositional changes of fecal microbiota.	The authors made no mention of adverse effects.

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Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Brigidi, et al. 2003 Highest dose tested: 5.58×10^{11} cfu/d of <i>Bifidobacterium</i> (<i>B. longum</i> , <i>B. infantis</i> , <i>B. breve</i>) plus other bacteria Longest exposure: 10 d Number participants receiving <i>B. breve</i>: 5	Objective: To develop a PCR assay in human fecal samples to detect <i>S. thermophilus</i> , <i>B. infantis</i> Y1, and <i>B. breve</i> Y8. Study type: Not stated Test Article: VSL#3 contained 3×10^{11} cfu/g of viable, lyophilized bacteria: 2.0×10^{11} cfu/g of <i>S. thermophilus</i> , 9.3×10^{10} cfu/g of <i>Bifidobacterium</i> (<i>B. longum</i> , <i>B. infantis</i> , <i>B. breve</i>) and 2.8×10^9 cfu/g of <i>Lactobacillus</i> (<i>L. acidophilus</i> , <i>L. casei</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. plantarum</i>).	Five healthy subjects ingested 250 g of yogurt daily, 5 healthy subjects received 6 g of VSL#3, and ten patients affected by IBD were treated with 6 g of VSL#3 per day for 10 d. Fecal samples were collected.	VSL#3 administration in healthy subjects tended to increase a higher fecal <i>S. thermophilus</i> concentration, versus baseline levels (no statistics). <i>B. breve</i> Y8 concentration decreased at 7 d in healthy subjects consuming VSL-3, but was stable for 6 d after discontinuing intake. <i>B. breve</i> Y8 was present in fecal samples taken from IBD patients at 2 mo.	The authors made no mention of adverse effects.

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Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Ishikawa, et al. 2002 Highest dose tested: 10×10^9 cfu/d of a mixture of <i>B. breve</i> , <i>B. bifidum</i> , and <i>L. acidophilus</i> YIT 0168 Longest exposure: 1 yr Number participants receiving <i>B. breve</i>: 11	<p><i>Objective:</i> To study the effect of a bifidobacteria-fermented milk with live bacteria on ulcerative colitis.</p> <p><i>Study type:</i> Randomized, controlled</p> <p><i>Test Article:</i> Bifidobacteria-fermented milk contained live <i>B. breve</i>, <i>B. bifidum</i>, and <i>L. acidophilus</i> YIT 0168 ($\geq 10^9$/100 mL bottle) and was from Yakult Co., Ltd.</p>	Subjects who had been diagnosed with ulcerative colitis at least one year previously were enrolled. Twenty-one subjects were randomly assigned to receive control ($n=10$) or the bifidobacteria-fermented milk containing live <i>B. breve</i> , <i>B. bifidum</i> , and <i>L. acidophilus</i> YIT 0168 (10×10^{10} /100 mL bottle) ($n=11$) per day for one year.	Fewer subjects in the treatment group experienced symptom exacerbation ($p=0.0075$), and fewer patients had 3 or more exacerbations overall ($p=0.009$), versus the control group. The cumulative exacerbation rate was also reduced in the treatment group versus placebo ($p=0.0184$). No difference was seen in colonoscopic findings at 1 yr between the groups. Significant increases in serum total protein and albumin levels were observed after probiotic supplementation ($p=0.02$ and 0.03 , respectively), versus control. At 1 yr, there was a reduction in the ratio of <i>Bacteroides vulgatus</i> in the probiotic group ($p<0.05$) and a decrease in fecal butyrate levels ($p<0.05$), versus control.	During month 2 of the 1 yr supplementation, one subject in the treatment group developed a coryza-like illness with abdominal pains. Supplementation was suspended for 2 wk, and then reinstated following treatment of the illness. The subject did not experience further abdominal pain, so this symptom was not thought to be related to the probiotic. The authors mention that no adverse effects were seen in any other subjects that might have been related to probiotic supplementation.

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Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<i>B. breve</i> combined with other species				
<p>Chang, et al. 2011</p> <p>Highest dose tested: <i>B. breve</i> plus other strains and functional ingredients (dose not stated)</p> <p>Longest exposure: 8 wk</p> <p>Number participants receiving <i>B. breve</i>: 53</p> <p style="writing-mode: vertical-rl; transform: rotate(180deg);">000122</p>	<p>Objective: To assess the effect of the consumption of a probiotics-supplemented yogurt on metabolic syndrome.</p> <p>Study type: Randomized, double-blind, placebo-controlled, parallel</p> <p>Test Article: The probiotic-supplemented yogurt contained <i>S. thermophilus</i>, <i>L. acidophilus</i>, <i>B. infantis</i> (these three organisms for fermentative purposes), plus fibersol-2 (resistant maltodextrin), FK-23 (<i>Enterococcus faecalis</i> FK-23), <i>Pinus densiflora</i> Seib et Zucc. extract, peptigen IF-3090 (whey protein hydroxylate[sic]), RGP-HC-90 (rice germ extract powder), <i>B. breve</i> and YQ-2 (<i>Yucca schidigera</i> and <i>Quillaja saponaria</i> extract. Control yogurt contained only <i>S. thermophilus</i>, <i>L. acidophilus</i>, and <i>B. infantis</i>. <i>B. breve</i> was from Chebigen Co., Ltd, Jeonju, Korea.</p>	<p>Volunteers aged 20-65 yr old were randomized to receive two bottles per day of either placebo ($n=48$; 15M/33F) or functional yogurt ($n=53$; 16M/37F), the latter of which contained numerous bacteria and functional substances, including <i>B. breve</i> (dose not stated) for 8 wk.</p> <p>Note: Control yogurt contained <i>S. thermophilus</i>, <i>L. acidophilus</i>, and <i>B. infantis</i>.</p>	<p>At 8 wk, the treatment group experienced a significant decrease in body weight ($P=0.006$), body mass index ($P=0.006$) and LDL-cholesterol ($P=0.044$), versus the control group.</p>	<p>There were no significant changes in waist circumference, systolic and diastolic blood pressure, fasting blood glucose, HbA1c, total cholesterol, HDL, or triglycerides between the two groups or within the groups. The authors made no mention of adverse effects.</p>

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Saggiaro 2004 Highest dose tested: Unknown <i>B. breve</i> BR0 + <i>L. plantarum</i> LP01 Longest exposure: 4 wk Number participants receiving <i>B. breve</i>: 24	Objective: To assess the effect of probiotics in patients having IBS. Study type: Randomized, controlled Test Article: A combination of either <i>B. breve</i> BR0 and <i>L. plantarum</i> LP01 or <i>L. plantarum</i> LP01 plus <i>L. acidophilus</i> LA02 was used.	Seventy patients (31M/39F; mean age 40 yr; range 26-64 yr) with IBS, according to Rome II criteria, were enrolled. Exclusions included organic disease on the basis of abdominal ultrasound and colonoscopy that was treated with various drugs without success. Subjects were randomized to receive 1) <i>L. plantarum</i> LP01 + <i>B. breve</i> BR0 (5×10^9 cfu/mL each) ($n=24$), 2) <i>L. plantarum</i> LP01 + <i>L. acidophilus</i> LA01 (5×10^9 cfu/mL each) ($n=26$) or 3) placebo powder containing starch ($n=20$) 2x/d for 4 wk. [Note: Total mL per day was not provided.]	Overall pain and symptoms scores in group 1 decreased at d 14 and d 28, versus baseline levels (no statistics).	The author made no mention of adverse effects.
Taheri, et al. 2011 Highest dose tested: <i>B. breve</i> Rosell-70 (dose not provided) plus other organisms Longest exposure: 4 wk Number participants receiving <i>B. breve</i>: 27	Objective: To evaluate the effects of probiotics in patients with microscopic colitis. Study type: Double-blind, placebo-controlled Test Article: Probiotics were from Optibac and contained a total of 5×10^9 cfu of a mixture of the following: <i>L. rhamnosus</i> Rosell-11, <i>L. acidophilus</i> Rosell-52, <i>B. longum</i> Rosell-175, <i>Lactococcus lactis</i> Rosell-1058, <i>B. breve</i> Rosell-70, and <i>B. bifidum</i> Rosell-71.	Fifty-two patients with microscopic colitis were enrolled in the study. Of these, 46 had lymphocytic colitis and six had collagenous colitis. All received 1 mg loperamide per day. Patients received two capsules twice daily, of either placebo ($n = 25$) or containing a probiotic mixture ($n = 27$) for 4 wk. Severity of abdominal pain, frequency of defecation and frequency of nocturnal defecation were assessed.	In the patients having lymphocytic colitis, abdominal pain and frequency of defecation (day and night) were significantly decreased in the probiotics group versus the placebo group ($p<0.001$).	Serious complications were not observed in either group, according to the authors. One patient in the probiotics group dropped out due to intensified diarrhea.

Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
<p>Eguchi, et al. 2011</p> <p>Highest dose tested: 45 mg <i>B. breve</i> Yakult + 60 mg <i>L. casei</i> Shirota + 45 g galacto-oligosaccharides/d</p> <p>Longest exposure: 2 d + 2 wk</p> <p>Number participants receiving <i>B. breve</i>: 25</p>	<p>Objective: To investigate the effect of synbiotics in patients receiving elective living donor liver transplantation.</p> <p>Study type: Prospective, randomized, controlled</p> <p>Test Article: The synbiotic was Yakult BL antifatulent and contained <i>B. breve</i> Yakult, <i>L. casei</i> Shirota (both from Yakult Honsha, Tokyo, Japan), and galacto-oligosaccharides (Oligomate 55; Yakult Honsha). The bacteria were from Yakult Honsha, Tokyo, Japan.</p>	<p>Fifty liver transplant recipients were enrolled in the study and randomly assigned to receive synbiotic therapy ($n=25$; 13M/12F; 33-66 yr) or none at all ($n=25$; 16M/9F; 25-68 yr) three times daily beginning 2 d prior to the elective living donor liver transplantation and continuing for 2 wk afterward (via tube jejunostomy or orally). Rates of infectious complications and patient survival were recorded and fecal samples were collected. The synbiotic contained 20 mg of living <i>L. casei</i> Shirota (cfu not given), 15 mg living <i>B. breve</i> Yakult (cfu not given), and 15 g of galacto-oligosaccharides per day three times per day.</p> <p>All patients received intravenous prophylaxis with amoxicillin and cefotiam for 4 d. Empiric therapy was initiated in the event of an infection and altered based on test results for resistance. Dual or triple immunosuppressive therapy was implemented and rituximab (anti-CD20 antibody) was used preoperatively in blood group-incompatible patients. At 24 h post-transplantation, all patients received an elemental diet enterally via tube jejunostomy.</p>	<p>There was no significant pattern of change of fecal bacteria species noted between the treatment groups. Infectious complications were significantly reduced in the synbiotic group versus the control group ($P<0.05$). The authors state that some infectious complication occurred after the termination of synbiotic therapy. There was no significant difference between the groups in other complications after transplantation.</p>	<p>There were no differences in the intensive care unit period, hospitalized period, and mortality rate between the synbiotic group and control group. There was no difference in the rejection rate, even though there were more blood group-incompatible transplant patients in the synbiotic group than in the control group.</p>

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Table 16. Human Clinical Studies of *Bifidobacterium breve* – Adults

Reference	Objective/Study Type/Test Article	Study Design	Outcome	Safety-Related Observations
Brigidi, et al. 2003 Highest dose tested: 5.58×10^{11} cfu/d of <i>Bifidobacterium</i> (<i>B. longum</i> , <i>B. infantis</i> , <i>B. breve</i>) plus other bacteria Longest exposure: 10 d Number participants receiving <i>B. breve</i>: 10	Objective: To develop a PCR assay in human fecal samples to detect <i>S. thermophilus</i> , <i>B. infantis</i> Y1, and <i>B. breve</i> Y8. Study type: Not stated Test Article: VSL#3 contained 3×10^{11} cfu/g of viable, lyophilized bacteria: 2.0×10^{11} cfu/g of <i>S. thermophilus</i> , 9.3×10^{10} cfu/g of <i>Bifidobacterium</i> (<i>B. longum</i> , <i>B. infantis</i> , <i>B. breve</i>) and 2.8×10^9 cfu/g of Lactobacillus (<i>L. acidophilus</i> , <i>L. casei</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. plantarum</i>).	Five healthy subjects ingested 250 g of yogurt daily, 5 healthy subjects received 6 g of VSL#3, and ten patients affected by inflammatory bowel diseases (IBD) were treated with 6 g of VSL#3 per day for 10 d. Fecal samples were collected.	VSL#3 administration in healthy subjects tended to increase a higher fecal <i>S. thermophilus</i> concentration, versus baseline levels (no statistics). <i>B. breve</i> Y8 concentration decreased at 7 d in healthy subjects consuming VSL#3, but was stable for 6 d after discontinuing intake. <i>B. breve</i> Y8 was present in fecal samples taken from IBD patients at 2 mo.	The authors made no mention of adverse effects.

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Appendix 1

BLASTN Analysis of 16S rDNA sequence of *Bifidobacterium breve* (B. breve) M-16V and B. breve ATCC15700.

Bifidobacterium breve M-16V 16s rDNA sequence for was obtained by direct sequencing. *B. breve* ATCC15700 16s rDNA sequence (Accession number AB006658) was obtained from the National Center for Biotechnology Information NCBI website. Sequences were aligned using the BLASTN software (Version 2.2.25+). Nucleotide differences are in bold and enlarged text. Although one mismatch was found, the 16S rDNA sequence of *B. breve* M-16V was highly homologous with that of *B. breve* ATCC15700. The results are provided below.

BLASTN 2.2.25+

Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.

ID: 1VS4XKAP113

Query= *B. breve* M-16V

Subject= *B. breve* ATCC15700

Length=1533

Sequences producing significant alignments:	Score (Bits)	E Value
dbj AB006658.1 <i>Bifidobacterium breve</i> gene for 16S rRNA, part...	2800	0.0

ALIGNMENTS

>dbj|AB006658.1| *Bifidobacterium breve* gene for 16S rRNA, partial sequence, strain:

ATCC 15700

Length=1520

Score = 2800 bits (1516), Expect = 0.0
Identities = 1518/1519 (99%), Gaps = 0/1519 (0%)
Strand=Plus/Plus

Query	12	TTCGATTCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGA	71
Sbjct	1	TTCGATTCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGA	60
Query	72	TCCATCGGGCTTTGC C TGGTGGTGGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCT	131
Sbjct	61	TCCATCGGGCTTTGC T TGGTGGTGGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCT	120

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Appendix 1

Sbjct	901	GGGCCCGCACAAAGCGGGGAGCATGCGGATTAATTGATGCAACGCGAAGAACCTTACCT	960
Query	972	GGGCTTGACATGTTCCCGACGATCCCAGAGATGGGGTTTCCCTTCGGGGCGGGTTCACAG	1031
Sbjct	961	GGGCTTGACATGTTCCCGACGATCCCAGAGATGGGGTTTCCCTTCGGGGCGGGTTCACAG	1020
Query	1032	GTGGTGCATGGTTCGTCGTCAGCTCGTGTGAGATGTTGGGTAAAGTCCCGCAACGAGC	1091
Sbjct	1021	GTGGTGCATGGTTCGTCGTCAGCTCGTGTGAGATGTTGGGTAAAGTCCCGCAACGAGC	1080
Query	1092	GCAACCCTCGCCCCGTGTTGCCAGCGGATTGTGCCGGGAACACGCGGGGACCGCCGGGG	1151
Sbjct	1081	GCAACCCTCGCCCCGTGTTGCCAGCGGATTGTGCCGGGAACACGCGGGGACCGCCGGGG	1140
Query	1152	TTAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCTTACGTCCAGGGCTTC	1211
Sbjct	1141	TTAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCTTACGTCCAGGGCTTC	1200
Query	1212	ACGCATGCTACAATGGCCGGTACAACGGGATGCGACAGTGCAGCTGGAGCGGATCCCTG	1271
Sbjct	1201	ACGCATGCTACAATGGCCGGTACAACGGGATGCGACAGTGCAGCTGGAGCGGATCCCTG	1260
Query	1272	AAAACCGGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGGCGGAGTCGCTA	1331
Sbjct	1261	AAAACCGGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGGCGGAGTCGCTA	1320
Query	1332	GTAATCGCGAATCAGCAACGTCGCGGTGAATGCGTTCCCGGGCCTTGTACACACCGCCCG	1391
Sbjct	1321	GTAATCGCGAATCAGCAACGTCGCGGTGAATGCGTTCCCGGGCCTTGTACACACCGCCCG	1380
Query	1392	TCAAGTCATGAAAGTGGGCAGCACCCGAAGCCGGTGGCCTAACCCCTTGC GGGAGGGAGC	1451
Sbjct	1381	TCAAGTCATGAAAGTGGGCAGCACCCGAAGCCGGTGGCCTAACCCCTTGC GGGAGGGAGC	1440
Query	1452	CGTCTAAGGTGAGGCTCGTGATTGGGACTAAGTCGTAACAAGGTAGCCGTACCGGAAGGT	1511
Sbjct	1441	CGTCTAAGGTGAGGCTCGTGATTGGGACTAAGTCGTAACAAGGTAGCCGTACCGGAAGGT	1500
Query	1512	GCGGCTGGATCACCTCCTT	1530
Sbjct	1501	GCGGCTGGATCACCTCCTT	1519

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APPENDIX 2A

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PROSAFE

Morinaga Milk Industry

NSL 1-83, 5-chome

228-8583

Zama-city, Kana

Japan

Antwerp, 25-aug-05

Dear Dr. Tomoko Yaeshima

The PROSAFE team is very grateful for providing strains to our project. Enclosed please find a first report summarising the results on species identification and antibiotic susceptibility testing. If enterococci were present in your set of strains, the possible presence of virulence genes was investigated by multiplex PCR. The PROSAFE project is currently conducting in-vitro experiments on typing and adherence, as well as in-vivo experiments in a mouse neutropenic model, and a rat endocarditis model. If any of your strains have been selected for either of these experiments, the results will be communicated to you in a second report in 2006.

The following bacterial culture is considered in the present report:

Genus-species as received

Bifidobacterium breve

Original Number

M-16V

Please carefully consider the following points :

When any of the data reported here are distributed to third parties or published by you, we request that a clear reference is made to the PROSAFE project i.e. by including the complete report and/or by referring to the project website <http://lmg.ugent.be/PROSAFE/>

The PROSAFE team intends to publish the results obtained with the strains considered in this report in peer-reviewed scientific journals without indicating the name of the company but with inclusion of the original strain number and strain history. Please indicate in the reply form if you wish that only the coded PRSF (PROSAFE) number should be used and not the original strain number.

Please indicate whether the strain considered in this report can be deposited in the public BCCM/LMG Bacteria Collection (Ghent University, Belgium; <http://www.belspo.be/bccm/lmg.htm>). Upon your agreement to deposit the strain, BCCM/LMG will contact you regarding an official material transfer agreement.

In order to verify that you have received this report, we kindly ask you to return the reply form by e-mail or fax.

If you have any questions concerning the results of your strain presented in the report, do not hesitate to contact the coordinator. Additional information concerning PROSAFE can also be found on our website: <http://lmg.ugent.be/PROSAFE/>

Sincerely yours, (b) (6)

(b) (6)

Prof. Dr. Herman Goossens,
PROSAFE Coordinator
on behalf of the PROSAFE Project Team

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*Investigations are performed to the best of our knowledge under the experimental conditions indicated.
Results cannot be used in judicial conflicts without written agreement of the PROSAFE coordinator*

Coordinator PROSAFE: Prof. Dr. Herman Goossens (University of Antwerp)
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A. CULTURES

The following bacterial culture was received:

Genus-species as received	Original Number: M-16V
<i>Bifidobacterium breve</i>	Depositor: Morinaga Milk Industry

B. AIM

Identification of the bacterial culture.

Antibiotic susceptibility testing of the bacterial culture.

C. TESTS PERFORMED

1. Recovery and purity check

Lactic acid bacteria were recovered on MRS agar or in MRS broth (Oxoid CM361), and incubated anaerobically at 37°C for 1 to 4 days.

2. Identification

BOX-PCR fingerprinting and cluster analysis

Cells were cultivated for 24h in MRS at 37°C and harvested from the fermentation liquor by centrifugation (13000 rpm, 15 min) and total DNA was extracted as described before (Gevers, D., G. Huys, and J. Swings. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol. Lett.* 205, 31-36, 2001). Rep-PCR fingerprinting was performed as described by Versalovic et al. (Versalovic J., M. Schneider, F. J. de Bruijn, and J. R. Lupski. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology* 5, 25-40, 1994) and as modified by Gevers et al., 2001). PCR amplifications were performed with the BOX primer BOXAIR (5'-CTACGGCAAGGCGACGCTGACG-3') was used (Masco, L., G. Huys, D. Gevers, L. Verbruggen, and J. Swings. Identification of *Bifidobacterium* species using rep-PCR fingerprinting. *Syst. Appl. Microbiol.* 26, 557-563, 2003). The PCR products were electrophoresed in an agarose gel. The BOX-PCR profiles were visualised after staining with ethidium bromide under ultraviolet light, followed by digital image capturing using a CCD camera. The resulting fingerprints were analysed using the BioNumerics V2.0 software package (Applied Maths, Ghent, Belgium). The similarity among digitised profiles was calculated using the Pearson correlation and an average linkage (UPGMA) dendrogram was derived from the profiles.

3. Antibiotic susceptibility testing

Susceptibility to the following antibiotics was tested:

penicillin (PEN), ampicillin (AMP), ampicillin/sulbactam (ASU), gentamicin (GEN) and streptomycin (STR) (including high-level resistance), vancomycin (VAN), teicoplanin (TPL), quinupristin/dalfopristin (Q/D), erythromycin (ERY), clindamycin (CLI), oxytetracycline (OTE), chloramphenicol (CMP), fusidic acid (FUS), trimethoprim (TMP), sulfamethoxazole/trimethoprim (SXT)

LSM broth supplemented with cysteine (0.3 g/l) served as nutrient medium for the MIC determinations of *Bifidobacterium* species in the pre-made micro titer plates. For the inoculation of these plates the test strains were freshly cultivated on modified Columbia agar* under anaerobic conditions (e.g. by AnaeroGen™, Oxoid) and at 37°C for 48 h. Single colonies of the corresponding strains were suspended in 5 ml saline up to an optical density of McFarland 0.5 standard. 10 µl of a 1:15 dilution of this suspension in saline served as inoculum for each well of the prepared microtiter plates with the different antibiotic concentrations and the growth control (without any antibiotic), respectively. Final inoculum of the bifidobacteria in the microtiter plates: about 105 bacteria/ml. The inoculated MIC test plates were then incubated at 37 °C in an anaerobic atmosphere for 48 h and the MICs were read as described before.

Investigations are performed to the best of our knowledge under the experimental conditions indicated.
Results cannot be used in judicial conflicts without written agreement of the PROSAFE coordinator

000141

* Modified Columbia Agar (pH 6.7; g/l aqua dest.):

Columbia Agar Base (e.g., CM331, Oxoid):		Supplementation with:	
Special peptone	23.0	Glucose	5.0
Soluble starch	1.0	Cystein hydrochloride	0.3
NaCl	5.0		
Agar	10.0		

(Klare et al., 2005, Applied and Environmental Microbiology (accepted))

Microbiological cut-offs:

Cut-offs defined on the basis of MIC distribution among a specific taxonomic group of the PRSF strain collection. The ultimate aim to define 'microbiological' cut-offs instead of 'clinical or pharmacokinetic/pharmacodynamic' cut-offs was to distinguish among 'intrinsic' and 'acquired' resistance traits. Depending on the taxonomic group studied, these cut-offs were specified at the genus or species level.

A minimum number of 10 strains of the same species is required to set microbiological cut-off values. However, for some of the species this number was not available in the PROSAFE collection. The antibiotic susceptibility results for these strains are mentioned as 'no cut-off values defined'.

Types of resistance

The type of resistance was defined as follows:

Intrinsic Resistance:

Natural or inherent resistance that is present in the wild type population of a given taxonomic group.

Acquired Resistance:

Type of resistance present in strains with MICs that are higher than the normal range of the MIC distribution of the wild type population of a given taxonomic group. This resistance usually originates from gene mutations or recombinations.

Inconclusive:

Due to insufficient information available, no clear distinction between intrinsic and acquired resistance can be made at this moment.

Cut-offs (based on final genus-species identification):

Bifidobacterium breve

Antibiotic	Cut-off
PEN	Inconclusive
AMP	Inconclusive
ASU	Inconclusive
GEN	Inconclusive
STR	Inconclusive
VAN	≤ 1
TPL	Inconclusive
Q/D	≤ 0.25
ERY	≤ 0.25
CLI	≤ 0.5
OTE	≤ 2
CMP	≤ 2
FUS	≤ 16
TMP	Inconclusive
SXT	Inconclusive

000142

D. RESULTS and CONCLUSIONS

Results

Original Number: M-16V

Identification

Depositor: Morinaga Milk Industry

Bifidobacterium breve

Antibiotic susceptibility testing:

Antibiotic	MIC
<i>PEN</i>	0.25
<i>AMP</i>	0.25
<i>ASU</i>	0.25
<i>GEN</i>	32
<i>STR</i>	32
<i>VAN</i>	0.5
<i>TPL</i>	≤ 0.125
<i>Q/D</i>	≤ 0.032
<i>ERY</i>	≤ 0.016
<i>CLI</i>	≤ 0.032
<i>OTE</i>	1
<i>CMP</i>	2
<i>FUS</i>	4
<i>TMP</i>	16
<i>SXT</i>	32
<i>LIN</i>	NT

Conclusions

1. Identification

Identification of the strain correct at the genus and at the species level

2. Antibiotic susceptibility testing

No acquired resistances detected

*Investigations are performed to the best of our knowledge under the experimental conditions indicated.
Results cannot be used in judicial conflicts without written agreement of the PROSAFE coordinator*

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APPENDIX 2B

000144

12 / XII

Numico
Bosrandweg 20
6704 PH Wageningen
Nederland

Antwerp, 20-okt-05

Dear Dr. Jan Knol

The PROSAFE team is very grateful for providing strains to our project. Enclosed please find a first report summarising the results on species identification and antibiotic susceptibility testing. If enterococci were present in your set of strains, the possible presence of virulence genes was investigated by multiplex PCR. The PROSAFE project is currently conducting in-vitro experiments on typing and adherence, as well as in-vivo experiments in a mouse neutropenic model, and a rat endocarditis model. If any of your strains have been selected for either of these experiments, the results will be communicated to you in a second report in 2006.

The following bacterial culture is considered in the present report:

Genus-species as received
Bifidobacterium breve

Original Number
EU-PS38

Please carefully consider the following points :

When any of the data reported here are distributed to third parties or published by you, we request that a clear reference is made to the PROSAFE project i.e. by including the complete report and/or by referring to the project website <http://img.ugent.be/PROSAFE/>

The PROSAFE team intends to publish the results obtained with the strains considered in this report in peer-reviewed scientific journals without indicating the name of the company but with inclusion of the original strain number and strain history. Please indicate in the reply form if you wish that only the coded PRSF (PROSAFE) number should be used and not the original strain number.

Please indicate whether the strain considered in this report can be deposited in the public BCCM/LMG Bacteria Collection (Ghent University, Belgium; <http://www.belspo.be/bccm/lmg.htm>). Upon your agreement to deposit the strain, BCCM/LMG will contact you regarding an official material transfer agreement.

In order to verify that you have received this report, we kindly ask you to return the reply form by e-mail or fax.

If you have any questions concerning the results of your strain presented in the report, do not hesitate to contact the coordinator. Additional information concerning PROSAFE can also be found on our website: <http://img.ugent.be/PROSAFE/>

(b) (6) (6)

Sincerely yours,

(b) (6)

Prof. Dr. Herman Goossens,
PROSAFE Coordinator
on behalf of the PROSAFE Project Team

*Investigations are performed to the best of our knowledge under the experimental conditions indicated.
Results cannot be used in judicial conflicts without written agreement of the PROSAFE coordinator*

000145

A. CULTURES

The following bacterial culture was received:

Genus-species as received	Original Number: EU-PS38
<i>Bifidobacterium breve</i>	Depositor: Numico

B. AIM

Identification of the bacterial culture.

Antibiotic susceptibility testing of the bacterial culture.

C. TESTS PERFORMED

1. Recovery and purity check

Lactic acid bacteria were recovered on MRS agar or in MRS broth (Oxoid CM361), and incubated anaerobically at 37°C for 1 to 4 days.

2. Identification

BOX-PCR fingerprinting and cluster analysis

Cells were cultivated for 24h in MRS at 37°C and harvested from the fermentation liquor by centrifugation (13000 rpm, 15 min) and total DNA was extracted as described before (Gevers, D., G. Huys, and J. Swings. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol. Lett.* 205, 31-36, 2001). Rep-PCR fingerprinting was performed as described by Versalovic et al. (Versalovic J., M. Schneider, F. J. de Bruijn, and J. R. Lupski. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology* 5, 25-40, 1994) and as modified by Gevers et al., 2001). PCR amplifications were performed with the BOX primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') was used (Masco, L., G. Huys, D. Gevers, L. Verbruggen, and J. Swings. Identification of *Bifidobacterium* species using rep-PCR fingerprinting. *Syst. Appl. Microbiol.* 26, 557-563, 2003). The PCR products were electrophoresed in an agarose gel. The BOX-PCR profiles were visualised after staining with ethidium bromide under ultraviolet light, followed by digital image capturing using a CCD camera. The resulting fingerprints were analysed using the BioNumerics V2.0 software package (Applied Maths, Ghent, Belgium). The similarity among digitised profiles was calculated using the Pearson correlation and an average linkage (UPGMA) dendrogram was derived from the profiles.

3. Antibiotic susceptibility testing

Susceptibility to the following antibiotics was tested:

penicillin (PEN), ampicillin (AMP), ampicillin/sulbactam (ASU), gentamicin (GEN) and streptomycin (STR) (including high-level resistance), vancomycin (VAN), teicoplanin (TPL), quinupristin/dalfopristin (Q/D), erythronycin (ERY), clindamycin (CLI), oxytetracycline (OTE), chloramphenicol (CMP), fustidic acid (FUS), trimethoprim (TMP), sulfamethoxazole/trimethoprim (SXT).

LSM broth supplemented with cystein (0.3 g/l) served as nutrient medium for the MIC determinations of *Bifidobacterium* species in the pre-made micro titer plates. For the inoculation of these plates the test strains were freshly cultivated on modified Columbia agar* under anaerobic conditions (e.g. by AnaeroGen™, Oxoid) and at 37°C for 48 h. Single colonies of the corresponding strains were suspended in 5 ml saline up to an optical density of McFarland 0.5 standard. 10 µl of a 1:15 dilution of this suspension in saline served as inoculum for each well of the prepared microtiter plates with the different antibiotic concentrations and the growth control (without any antibiotic), respectively. Final inoculum of the bifidobacteria in the microtiter plates: about 105 bacteria/ml. The inoculated MIC test plates were then incubated at 37 °C in an anaerobic atmosphere for 48 h and the MICs were read as described before.

Investigations are performed to the best of our knowledge under the experimental conditions indicated.
Results cannot be used in judicial conflicts without written agreement of the PROSAFE coordinator

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* *Modified Columbia Agar (pH 6.7; g/l aqua dest.):*

<i>Columbia Agar Base (e.g., CM331, Oxoid):</i>		<i>Supplementation with:</i>	
<i>Special peptone</i>	23.0	<i>Glucose</i>	5.0
<i>Soluble starch</i>	1.0	<i>Cystein hydrochloride</i>	0.3
<i>NaCl</i>	5.0		
<i>Agar</i>	10.0		

(Klare et al., 2005, *Applied and Environmental Microbiology* (accepted))

Microbiological cut-offs:

Cut-offs defined on the basis of MIC distribution among a specific taxonomic group of the PRSF strain collection. The ultimate aim to define 'microbiological' cut-offs instead of 'clinical or pharmacokinetic/pharmacodynamic' cut-offs was to distinguish among 'intrinsic' and 'acquired' resistance traits. Depending on the taxonomic group studied, these cut-offs were specified at the genus or species level.

A minimum number of 10 strains of the same species is required to set microbiological cut-off values. However, for some of the species this number was not available in the PROSAFE collection. The antibiotic susceptibility results for these strains are mentioned as 'no cut-off values defined'.

Types of resistance

The type of resistance was defined as follows:

Intrinsic Resistance:

Natural or inherent resistance that is present in the wild type population of a given taxonomic group.

Acquired Resistance:

Type of resistance present in strains with MICs that are higher than the normal range of the MIC distribution of the wild type population of a given taxonomic group. This resistance usually originates from gene mutations or recombinations.

Inconclusive:

Due to insufficient information available, no clear distinction between intrinsic and acquired resistance can be made at this moment.

Cut-offs (based on final genus-species identification):

Bifidobacterium breve

<u>Antibiotic</u>	<u>Cut-off</u>
<i>PEN</i>	<i>Inconclusive</i>
<i>AMP</i>	<i>Inconclusive</i>
<i>ASU</i>	<i>Inconclusive</i>
<i>GEN</i>	<i>Inconclusive</i>
<i>STR</i>	<i>Inconclusive</i>
<i>VAN</i>	≤ 1
<i>TPL</i>	<i>Inconclusive</i>
<i>Q/D</i>	≤ 0.25
<i>ERY</i>	≤ 0.25
<i>CLI</i>	≤ 0.5
<i>OTE</i>	≤ 2
<i>CMP</i>	≤ 2
<i>FUS</i>	≤ 16
<i>TMP</i>	<i>Inconclusive</i>
<i>SXT</i>	<i>Inconclusive</i>

Investigations are performed to the best of our knowledge under the experimental conditions indicated. Results cannot be used in judicial conflicts without written agreement of the PROSAFE coordinator

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D. RESULTS and CONCLUSIONS

Results

Original Number: EU-PS38

Identification

Depositor: Numico

Bifidobacterium breve

Antibiotic susceptibility testing:

Antibiotic	MIC
<i>PEN</i>	0.125
<i>AMP</i>	0.125
<i>ASU</i>	0.125
<i>GEN</i>	128
<i>STR</i>	128
<i>VAN</i>	0.5
<i>TPL</i>	≤ 0.125
<i>Q/D</i>	0.125
<i>ERY</i>	0.25
<i>CLI</i>	0.125
<i>OTE</i>	2
<i>CMP</i>	2
<i>FUS</i>	8
<i>TMP</i>	64
<i>SXT</i>	128
<i>LIN</i>	NT

Conclusions

1. Identification

Identification of the strain correct at the genus and at the species level.

2. Antibiotic susceptibility testing

No acquired resistances detected.

*Investigations are performed to the best of our knowledge under the experimental conditions indicated.
Results cannot be used in judicial conflicts without written agreement of the PROSAFE coordinator*

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APPENDIX 2C

000149

Identification of strain NR1
 (DSM ID 05-1030)

16.02.2006

Bifidobacterium breve

Properties of the strain

Rods	+
width μm	0.6-0.8
length μm	2.5-3.5
Gram-reaction	+
Oxidase	-
Katalase	-
Acid from	
Trehalose	-
Melibiose	+
Amygdalin	-
L-Arabinose	-
Mannit	+
Sorbit	+
Melezitose	-
Raffinose	+
Rhamnose	-
Galactose	+
D-Glucose	+
Lactose	+
Ribose	+
Esculin	+
Cellobiose	-
Maltose	+
Mannose	+
Fructose	+
Growth at 15°C	-
45°C	-
Arginindihydrolase	-

RESULT: strain NR1

= *Bifidobacterium breve*

The profile of the cellular fatty acids contains the typical components for this group of bacteria.

The partial sequences of the 16S rDNA shows a similarity of 100% to *Bifidobacterium breve*.

The physiological tests almost confirm this result.



Volume: DATA File: E05C084.98A Seq Counter: 7 ID Number: 14271
 Type: Samp Bottle: 11 Method: TSBA40
 Created: 08.12.2005 14:23:42
 Sample ID: UN-V-05-1030-NR1-BIBT-BREVE(M.11,37C,1d)

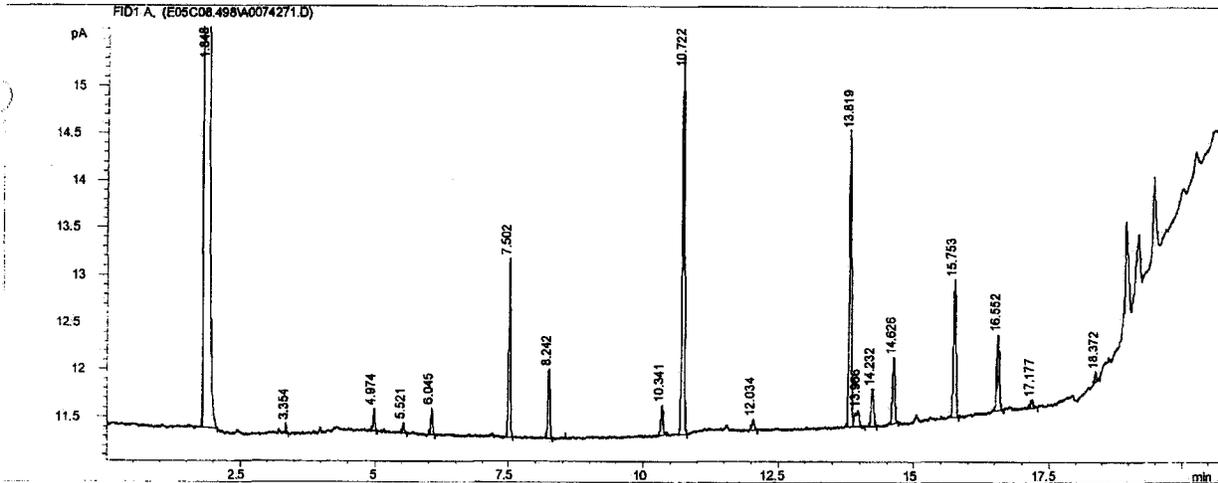
RT	Response	Ar/Ht	RFact	ECL	Peak Name	-Percent	Comment1	Comment2
1.848	3.538E+8	0.027	----	7.021	SOLVENT PEAK	----	< min rt	
3.354	372	0.027	1.176	10.000	10:0	0.66	ECL deviates 0.000	Reference 0.001
4.974	940	0.033	1.067	12.001	12:0	1.51	ECL deviates 0.001	Reference 0.000
5.521	409	0.032	1.047	12.474	unknown 12.484	0.64	ECL deviates -0.010	
6.045	1223	0.035	1.029	12.927	13:1 AT 12-13	1.89	ECL deviates -0.009	
7.502	8962	0.037	0.994	14.000	14:0	13.36	ECL deviates 0.000	Reference -0.001
8.242	3546	0.039	0.981	14.478	Sum In Feature 1	5.22	ECL deviates 0.000	15:1 ISO I/13:0 3OH
10.341	1745	0.043	0.951	15.772	16:1 w9c	2.49	ECL deviates -0.002	
10.722	22053	0.044	0.947	15.999	16:0	31.32	ECL deviates -0.001	Reference -0.002
12.034	632	0.044	----	16.753				
13.819	18231	0.044	0.920	17.767	18:1 w9c	25.15	ECL deviates -0.002	
13.966	1646	0.065	0.919	17.850	18:1 w6c	2.27	ECL deviates -0.008	
14.232	2532	0.048	0.917	18.000	18:0	3.48	ECL deviates 0.000	Reference 0.000
14.626	4256	0.049	----	18.224				
15.753	8841	0.047	0.907	18.864	Sum In Feature 7	12.03	ECL deviates -0.003	19:0 CYCLO w10c/19w6
16.552	4961	0.051	----	19.321				
17.177	613	0.051	----	19.680				
18.372	439	0.033	----	20.369				
----	3546	----	----	----	Summed Feature 1	5.22	> max rt	
							15:1 ISO H/13:0 3OH	13:0 3OH/15:1 i i/H
							15:1 ISO I/13:0 3OH	
----	8841	----	----	----	Summed Feature 7	12.03	un 18.846/19:1 w6c	19:1 w6c/.846/19cy
----	----	----	----	----			19:0 CYCLO w10c/19w6	

ECL Deviation: 0.005
 Total Response: 80959
 Percent Named: 87.08%

Reference ECL Shift: 0.001 Number Reference Peaks: 5
 Total Named: 70498
 Total Amount: 66675

Matches:

Library	Sim Index	Entry Name
TSBA40 4.10	0.042	Lactobacillus-parabuchneri



APPENDIX 2D

Dr. R. D. Wind
NUMICO RESEARCH B.V.
Biomedical Research Department
Bosrandweg 20
PO Box 7005
6700 CA Wageningen
Nederland

GENT, 15 maart 2006

YOUR REF.: Order brief dd. 28/11/05 (MTA ref. MTA.BCCM/LMG.051115.B)
E-mail dd. 09/03/06
OUR REF.: **Culturen ontvangen 29/11/05**
E-mail partiële resultaten 22/12/05
E-mail resultaten dd. 09/03/06
E-mail dd. 13/03/06

DETAILED REPORT

Geachte Dr. Wind,

Ingesloten vindt u het rapport betreffende de identificatie dmv DNA fingerprinting (FAFLP) van de volgende culturen:

***Bifidobacterium breve* NumRES TD1 = ID9054**
***Bifidobacterium longum* NumRES 9 = ID9055**
***Lactobacillus plantarum* NumRES 10 = ID9056**
***Lactobacillus rhamnosus* NumRES 1 = ID9057**
***Lactobacillus plantarum* NumRES 8 = ID9058**

Met vriendelijke groeten,

Ilse Cleenwerck voor
Dr. D. Janssens
BCCM/LMG Manager

(FACTUUR INGESLOTEN)

000153

Identification of 5 bacterial cultures by DNA fingerprinting (FAFLP)

report 14/03/06

performed by order of:

NUMICO RESEARCH B.V.
Biomedical Research Department
Bosrandweg 20
PO Box 7005
6700 CA Wageningen
Nederland

000154

1.- MATERIAL

Five cultures were received on 29/11/05 on agar slants from DR. R. D. WIND, NUMICO RESEARCH B.V., The Netherlands. Following identification numbers were allocated:

***Bifidobacterium breve* NumRES TD1 = ID9054**
***Bifidobacterium longum* NumRES 9 = ID9055**
***Lactobacillus plantarum* NumRES 10 = ID9056**
***Lactobacillus rhamnosus* NumRES 1 = ID9057**
***Lactobacillus plantarum* NumRES 8 = ID9058**

2.- AIM OF THE STUDY

Identification using DNA fingerprinting (FAFLP technique)

3.- TESTS PERFORMED

► Cultivation and purity check

• The cultures were recovered and checked for purity after incubation at 37°C for 24 or 48 h under anaerobic conditions on Columbia Blood Agar Base (Oxoid CM331) supplemented with 5% sheep blood (ID9054 and ID9055) or MRS (Oxoid CM361) (ID9056, ID9057 and ID9058). ID9054, ID9055, ID9056 and ID9057 showed a homogeneous growth. ID9058 showed smoother and rougher colony types that were unstable upon subculturing.

► Basic bacteriological tests

Following elementary bacteriological tests were performed: cell morphology, gram stain, oxidase and catalase reaction.

► DNA fingerprinting using FAFLP

AFLP™ is a PCR based technique for whole genome DNA fingerprinting via the selective amplification of restriction fragments (Vos *et al.*, Nucleic Acids Research 23: 4407-4414 (1995)).

• **DNA was prepared** using the method of Gevers *et al.* (Gevers, D., G. Huys, and J. Swings. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol. Lett. 205, 31-36, 2001).

• Purified total DNA was **digested** by two restriction enzymes, a 4-base cutter and a 6-base cutter. In this way, only a limited number of fragments with two different ends and of suitable size for efficient PCR are obtained. Small ds DNA molecules (15-20 bp) containing one compatible end were **ligated** to the appropriate 'sticky end' of the restriction fragments. Both **adaptors** are restriction halvesite-specific and have different sequences. These adaptors serve as binding sites for PCR primers. In the current analyses, following **restriction enzymes** and **adaptors** were used:

```
restriction enzyme: EcoR I [hexacutter]
Adaptor:           5' -CTCGTAGACTGCGTACC-3'
                   3' -CTGACGCATGGTTAA-5'

restriction enzyme: Taq I [tetracutter]
Adaptor:           5' -GACGATGAGTCCTGAC-3'
                   3' -TACTCAGGACTGGC-5'
```

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000156

- **Selective amplification** of some of the restriction fragments: PCR primers were specifically hybridized with the adaptor ends of the restriction fragments. Since the primers contain at their 3'-end one or more so-called 'selective bases' that extend beyond the restriction site into the fragment, only those restriction fragments that have the appropriate complementary sequence adjacent to the restriction site will be amplified.

Following **primercombination** was used:

Primercombination	
E01:	5' -GACTGCGTACCAATTCA-3'
T01:	5' -CGATGAGTCCTGACCGAA-3'

- **PCR products were separated** according to their length on a high resolution polyacrylamide gel using a DNA sequencer (ABI 377). Fragments that contain an adaptor specific for the restriction halfsite created by the 6-bp cutter are **visualised** due to the 5'-end labeling of the corresponding primer with the fluorescent dye FAM.
- The resulting electrophoretic patterns were tracked and normalized using the GeneScan 3.1 software (Applied Biosystems, USA). Normalized tables of peaks, containing fragments of 50 to 536 base pairs, were transferred into the BioNumerics™ 4.5 software (Applied Maths, Belgium). For **numerical analysis**, a data interval was delineated between the 75- and 500-bp bands of the internal size standard. Clustering of the patterns was done using the Dice coefficient and the UPGMA algorithm. The profiles were compared with the reference profiles of the lactic acid bacteria taxa (including bifidobacteria) as currently available in our database.

4.- RESULTS AND CONCLUSIONS

Results and conclusions are given on next pages.

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IDENTIFICATION ID9054**▶ Basic bacteriological tests**

Cell morphology: rod (0,8 x 2-3 µm); single, pairs, V-form; nonmotile; no spores
Gram stain: positive
Oxidase reaction: negative
Catalase reaction: negative

▶ Identification using DNA fingerprinting (FAFLP): Clusteranalysis with all available profiles of reference strains in our current 'LAB' database identified the strain as *Bifidobacterium breve*.

The computer-generated profiles and the dendrogram of the clusteranalysis are given on page 6.

CONCLUSION**▶ Identification ID9054: *Bifidobacterium breve*****IDENTIFICATION ID9055****▶ Basic bacteriological tests**

Cell morphology: rod (0,8 x 2-3 µm); single, pairs, v-form; nonmotile; no spores
Gram stain: positive
Oxidase reaction: negative
Catalase reaction: negative

▶ Identification using DNA fingerprinting (FAFLP): Clusteranalysis with all available profiles of reference strains in our current 'LAB' database identified the strain as *Bifidobacterium longum* biotype longum.

The computer-generated profiles and the dendrogram of the clusteranalysis are given on page 6.

CONCLUSION**▶ Identification ID9055: *Bifidobacterium longum* biotype longum**

IDENTIFICATION ID9056▶ **Basic bacteriological tests**

Cell morphology: rod (1 x 1,5-2,5 µm); single, pairs; nonmotile; no spores
Gram stain: positive
Oxidase reaction: negative
Catalase reaction: negative

▶ **Identification using DNA fingerprinting (FAFLP): Clusteranalysis** with all available profiles of reference strains in our current 'LAB' database identified the strain as *Lactobacillus plantarum*.

The computer-generated profiles and the dendrogram of the clusteranalysis are given on page 6.

CONCLUSION▶ **Identification ID9056: *Lactobacillus plantarum*****IDENTIFICATION ID9057**▶ **Basic bacteriological tests**

Cell morphology: rod (1 x 2-3 µm); single, pairs, chains; nonmotile; no spores
Gram stain: positive
Oxidase reaction: negative
Catalase reaction: negative

▶ **Identification using DNA fingerprinting (FAFLP): Clusteranalysis** with all available profiles of reference strains in our current 'LAB' database identified the strain as *Lactobacillus rhamnosus*.

The computer-generated profiles and the dendrogram of the clusteranalysis are given on page 7.

CONCLUSION▶ **Identification ID9057: *Lactobacillus rhamnosus***

000159

IDENTIFICATION ID9058**▶ Basic bacteriological tests**

Cell morphology: rod (1 x 2-3 µm); single, pairs; nonmotile; no spores
Gram stain: positive
Oxidase reaction: negative
Catalase reaction: negative

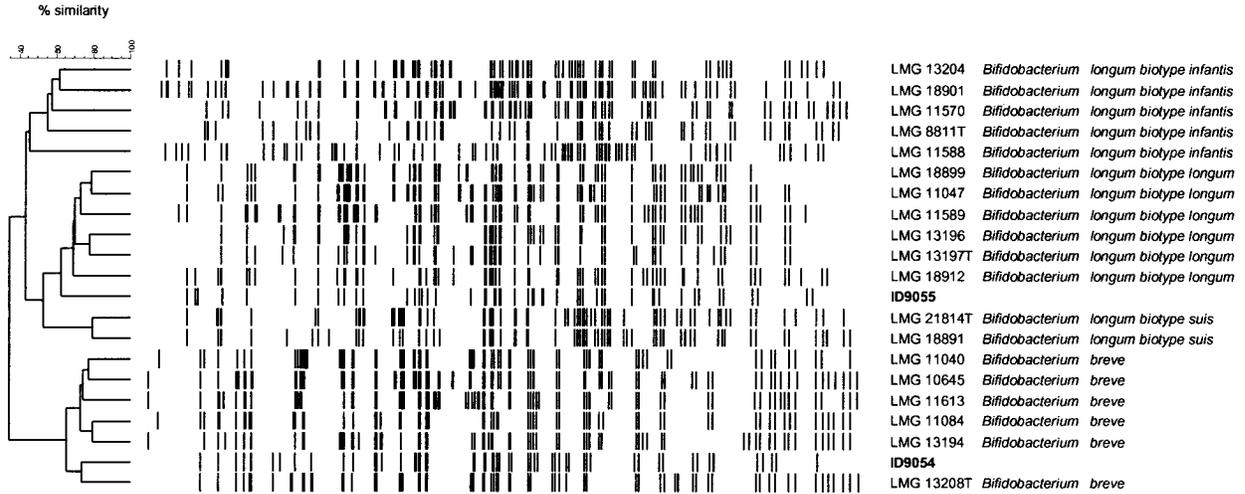
- ▶ Identification using DNA fingerprinting (FAFLP): Clusteranalysis** with all available profiles of reference strains in our current 'LAB' database identified the strain as *Lactobacillus plantarum*.

The computer-generated profiles and the dendrogram of the clusteranalysis are given on page 6.

CONCLUSION

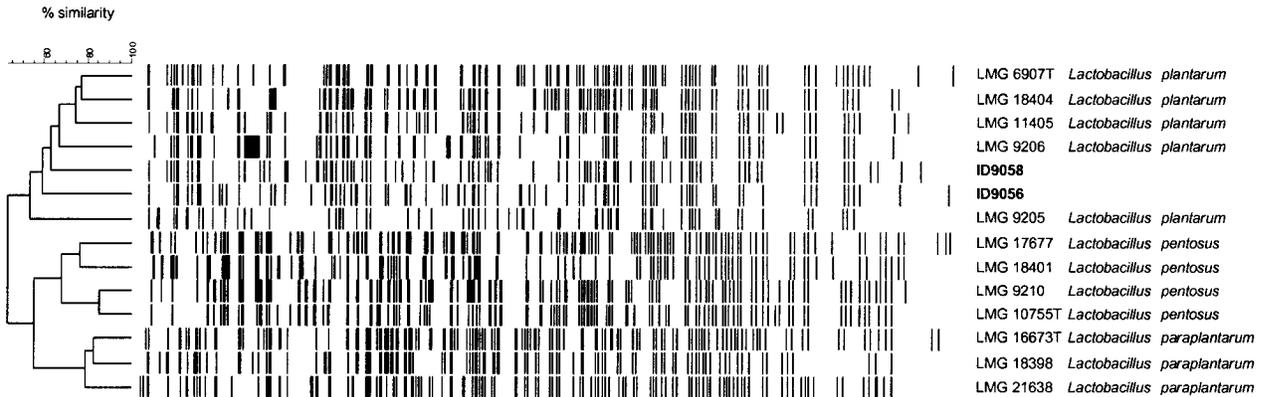
- ▶ Identification ID9058: *Lactobacillus plantarum***

COMPUTER-GENERATED PROFILES AND DENDROGRAM OF THE CLUSTERANALYSIS OF FAFLP DNA FINGERPRINTS FROM REFERENCE STRAINS OF BIFIDOBACTERIUM LONGUM AND BIFIDOBACTERIUM BREVE AND FROM ID9054 AND ID9055



Type strains are labelled with T.

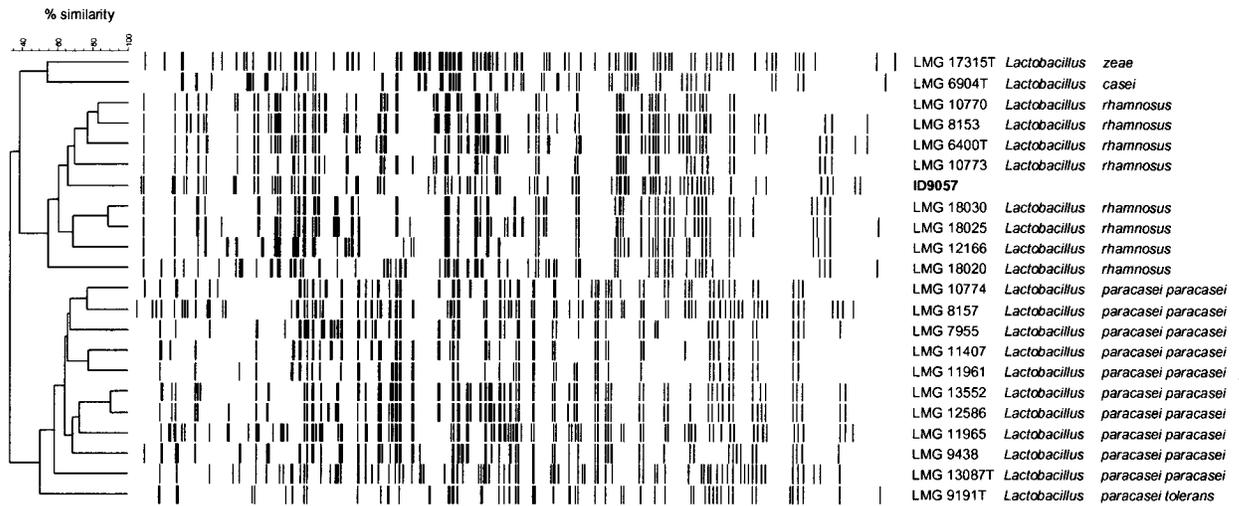
COMPUTER-GENERATED PROFILES AND DENDROGRAM OF THE CLUSTERANALYSIS OF FAFLP DNA FINGERPRINTS FROM REFERENCE STRAINS OF LACTOBACILLUS PLANTARUM AND PHYLOGENETICALLY RELATED SPECIES AND FROM ID9056 AND ID9058



Type strains are labelled with T.

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COMPUTER-GENERATED PROFILES AND DENDROGRAM OF THE CLUSTERANALYSIS OF
FAFLP DNA FINGERPRINTS FROM REFERENCE STRAINS OF *LACTOBACILLUS RHAMNOSUS*
AND PHYLOGENETICALLY RELATED SPECIES AND FROM ID9057



Type strains are labelled with T.

APPENDIX 3A

Certificate Number JP07/00064HP

SGS

The management system of
MORINAGA MILK INDUSTRY CO., LTD.
Tone Plant
Functional Materials Production Department



4013-1, Uchimoriyomachi, Joso-shi,
Ibaraki Pref. Japan 303-0043

has been assessed and certified as meeting the requirements of

HACCP Codex Alimentarius
Hazard Analysis and Critical Control Point (HACCP)
System and Guidelines for its application
Annex to CAC/RCP-1-1969, Rev. 4(2003)

For the following activities

From acceptance of raw materials to shipment of products

This certificate is valid from 3 September, 2007 until 2 September, 2010
Issue#01. Certified since 3 September, 2007

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Authorised by

(b) (6)

Masahiro Mukai



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APPENDIX 3B

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Products with HACCP Plan

Ref No. JP07/00064HP

1. Bifidobacteria and Lactic acid bacteria powder products



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Analytical Methods

I. Genotypic Identification

A. Guanine and cytosine content

Guanine (G) and cytosine (C) content was determined by thermal melting point analysis using DNA harvested from *Bifidobacterium breve* (*B. breve*) M-16V and *B. breve* ATCC15700 as described (MARMUR and DOTY, 1962).

B. Genomic homology of *B. breve* M-16V and other strains of bifidobacteria

The genomic homology of *B. breve* M-16V was determined using a colorimetric microplate hybridization method as described (Yaeshima et al., 1996). Briefly, 96 well plates were coated with DNA isolated from *B. breve* M-16 and *B. breve* ATCC15700, hybridized with DNA isolated from the different bifidobacterial species, and the amount of bound DNA was quantified using a colorimetric assay.

C. 16S rDNA sequencing

16S rDNA sequence for *B. breve* M-16V was obtained from direct sequencing. The 16S rDNA sequence of *B. breve* ATCC15700 (accession number AB006658) was obtained from National Center for Biotechnology Information NCBI website. Sequences were aligned using the BLASTN software (Version 2.2.25+).

D. Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) Analysis

RAPD-PCR is performed as described (Xiao et al., 2010) using DNA extracted from *B. breve* M-16V grown from finished products.

E. Genomic Sequence

The genomic sequence of *B. breve* M-16V was determined to a depth of approximately 7x using ABI 3730 capillary sequencer. The gaps were filled by primer-walking and sequencing the PCR products. The entire sequence was assembled using PHAP and PGA assemblers. The genome of M-16V contains 2,269,379 base pairs with a GC content of 58.9%.

II. Phenotypic Identification

A. Carbohydrate fermentation pattern

The carbohydrate fermentation pattern of *B. breve* M-16V and *B. breve* ATCC 15700 were determined using API 50 CH strips (bioMérieux, Inc.) as described (Boyd et al., 2005).

B. Fructose 6-phosphate phosphoketolase activity

Fructose 6-phosphate phosphoketolase activity of *B. breve* M-16V and the type strain *B. breve* ATCC 15700 were determined as described (Scardovi, 1986).

C. Carbohydrate fermentation products

Fermentation products were determined by culturing *B. breve* M-16V in peptone-yeast medium [peptone (5 g/l), tryptone (5 g/l), yeast extract (10 g/l), vitamin K1 (3 drops), cysteine-HCl (0.5 g/l) and 40 ml of salt solution containing (MgSO₄ (0.2g/l), CaCl₂ (0.2 g/l), K₂HPO₄ (1 g/l), KH₂PO₄ (1 g/l), NaHCO₃ (10 g/l), and NaCl (2 g/l))] and the carbon sources D-(+)-glucose (10 g/l), D-lactose (10 g/l), maltodextrin (10 g/l), galactooligosaccharide (GOS; 10 g/l), or GOS/fructooligosaccharide (FOS) (10 g/l) for 48 h at 37°C. Samples were harvested at various time points and D- and L-lactate, acetic acid, propionic acid, (iso)butyric acid, and isovaleric acid were determined using L- and D-lactic acid kits (Enzyplus) and gas chromatography. The amount of D- and L-lactate was also determined by high performance liquid chromatography.

D. Resistance to bile acids

The resistance of *B. breve* M-16V to bile acids was evaluated by anaerobically culturing *B. breve* M-16V and the type strain *B. breve* ATCC 15700 at 37°C in GAM broth (Nissui Pharmaceutical Co., Ltd.) containing 0.1% and 0.3% bile extract (Oxgall, Wako Pure Chemical Industries). The number of viable cells was then determined 24 hr later by plating an aliquot of the resulting culture on BL agar medium (Nissui Pharmaceutical Co., Ltd.), incubating the plates anaerobically at 37°C, and counting the number of colonies.

III. Quality Control and Product Specifications

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A. Cell morphology

Morphology is determined microscopically.

B. Cell density

Cell density is determined by visually inspecting the turbidity of the culture.

C. Appearance, odor, taste, and the presence of foreign bodies

Twenty grams M-16V powder is spread on a test table and checked for color, odor, taste, and the presence of foreign bodies.

D. Anaerobic colony forming units (cfu)

Anaerobic colony forming units (cfu) is determined by enumeration as described (Muto et al., 2010). One gram of M-16V or M-16V Type T finished products is suspended in 99 ml Mitsuoka's Buffer and one ml of the suspension is serially diluted with 9 ml Mitsuoka's Buffer without Bacto agar. One ml of the dilutions are then added to Petri dishes and mixed with melted Reinforced clostridial agar (OXOID). After solidification, the Petri dishes are incubated anaerobically for 72 h at 37°C. The colonies are counted and the anaerobic CFU is determined by multiplying the number of colonies by the dilution factor to determine the number of cfu/g.

E. Aerobic cfu

Ten grams of M-16V or M-16V Type T finished products are suspended in 90 ml sterilized saline. One hundred µl or 1 ml of the suspension are then mixed with melted Standard Plate Count Agar, which is added to Petri dishes incubated aerobically for 48 h at 35 °C. The colonies are then counted and multiplied by the appropriate dilution factor to determine the number of cfu/g.

F. Enterobacteriaceae

Ten grams of M-16V or M-16V Type T finished products are suspended in 100 ml of sterilized saline. One hundred µl of the suspension are then mixed with melted Violet Red Bile Dextrose (VRBD) agar, which is poured into Petri dishes and incubated for 24 h at 35 °C. If colonies appear, the finished product is deemed positive.

G. Enterococci

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Ten grams of M-16V or M-16V Type finished products are suspended in 90 ml of sterilized saline. One hundred μ l of the suspension is then added to azide citrate broth and cultured for 48 hours at 35°C. If the culture is turbid after 48 h, the finished product is deemed positive for enterococci.

H. *Escherichia coli*

One gram of M-16V or M-16V type T finished products are suspended in 99 ml Enterobacteriaceae Enrichment (EE) broth and incubated for 24 h at 35°C. ECB (*Escherichia coli* (*E. coli*) broth) is then inoculated with 1 ml of the M-16V/EE culture and incubated for an additional 24 hours at 44.5°C. If the culture is turbid and gas is generated, the finished product is deemed positive for *E. coli*.

I. *Clostridium perfringens*

Ten grams of M-16V or M-16V Type T finished products are suspended in 90 ml sterilized saline. Ten ml or 1 ml of the suspension is mixed with 15 ml melted Clostridia Count agar and poured into a porch. The porch is sealed, cooled, and cultured anaerobically for 24 h at 35°C. If black colonies appear, the finished product is deemed positive for *Clostridium perfringens*.

J. *Staphylococcus aureus*

Ten grams of M-16V or M-16V Type T finished products are suspended in 90 ml sterilized saline. One hundred μ l of the suspension is mixed with melted York Mannitol Salt Agar with egg yolk, poured into Petri dishes, and incubated for 48 h at 35 °C. If colonies appear, the finished product is deemed positive for *Staphylococcus aureus*.

K. Mold and yeast

Ten grams of M-16V or M-16V Type T finished products are suspended in 90 ml sterilized saline. Five 2 ml aliquots of the suspension are mixed independently with melted Potato dextrose agar medium and incubated for 5 days at 25 °C. The colonies are then counted and multiplied by appropriate dilution factor to determine the number of cfu/g.

L. *Salmonella*

Appendix 4

Twenty-five gram of M-16V or M-16V Type T finished products are suspended in 225 ml of sterilized buffered peptone water (BPW) and incubated for 18 h at 37°C. A loopful of this mixture is then added to selenite enrichment broth and incubated for an additional 24 h at 37°C. Finally, a loopful of the selenite enrichment culture is plated on deoxycholate hydrogen sulfide lactose (DHL) agar and cultured for 24 h at 37°C. If black colonies develop, they are picked and transferred to a Triple Sugar Iron agar (TSI) slant and a Lysine-indole-motility agar (LIM) slant. The slants are then incubated for 24 h at 37°C and if black bubbles appear and the upper part of the TSI agar slant is yellow, and cell growth is apparent in the LIM slant without a change in color, the finished product is deemed positive for *Salmonella*.

M. *Cronobacter sakazakii*

Twenty-five gram of M-16V or M-16V Type T finished products are suspended in 225 ml BPW and incubated for 18 h at 37°C. A loopful of the mixture is then transferred to EE broth and incubated for an additional 24 h at 37°C. Finally, a loopful of the EE culture is transferred to plate containing VRBD agar and incubated for 24 h at 37°C. If colonies appear, they are picked and transferred to a plate containing tryptone soy (TS) agar and cultured for 72 h at 37°C. Then, if yellow colonies appear on the TS agar plates, they are picked and analyzed by the API-20 kit (bioMerieux, Inc) following the manufacturer's protocol. If the API test is positive, the finished product is deemed positive *Cronobacter. sakazakii*.

N. *Bacillus cereus*

Ten grams of M-16V or M-16V Type T finished products are suspended in 90 ml sterilized saline. Five 200 µl aliquots of the suspension are then independently inoculated NGKG agar plates and incubated at 48 h at 37°C. Colonies are then counted and multiplied by the appropriate dilution factor to determine the number of *Bacillus cereus* cfu/g.

O. Lead

Lead content is determined using an Agilent 7500CE inductively coupled plasma mass spectrophotometer (ICP-MS).

P. Arsenic and heavy metals

The presence of arsenic and heavy metals is determined by using a PANalytical Epsilon 5 X-ray Fluorescence system.

Q. Loss on Drying

Moisture content is determined by comparing the weight of a small amount of finished product before and after heating at 105°C for 4 h.

R. Casein and β -lactoglobulin content

Two grams of M-16V or M-16V Type T finished products are suspended in 18 ml of sample extraction solution. Casein and β -lactoglobulin content are determined by enzyme-linked immunosorbant assays (ELISAs) using the Morinaga FASPEK kit.

S. Survivability

The survivability of *B. breve* M-16V in M-16V and M-16V Type T was determined as described (Abe et al., 2009a)

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

IV. Safety Assessment

A. *In vitro* safety studies

1. Antibiotic sensitivity

Morinaga Milk Industry determined the antibiotic sensitivity of *B. breve* M-16V according to ISO 13969/IDF 183. Danone Research determined the antibiotic sensitivity as described (Klare et al., 2005).

2. Presence of plasmids

Plasmids were isolated from *Lactobacillus paracasei* and *B. breve* M-16V as described (Anderson and McKay, 1983).

3. D-lactic acid production

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Appendix 4

The production of lactic acid isomers by *B. breve* M-16V, *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2772, and *Lactobacillus rhamnosus* (*L. rhamnosus*) LW744 was determined by culturing the bacteria in MRS medium for 48 h at 37°C and quantifying the lactic acid isomers in the medium using the Enzyplus EZA-890 and 889 kits from Rasio Diagnostics. The production of lactic acid isomers by *B. breve* M-16V and *B. breve* ATCC 15700 was also determined using the F-Kit TC D-/L-Lactic Acid from Roche Diagnostics.

4. Bile salt deconjugation

Bile salt deconjugation was analyzed by culturing *B. breve* M-16V anaerobically for 16 h at 37 °C in GAM broth (Nissui Pharmaceutical Co., Ltd.) containing 0.1 mM cholic, taurocholic, glycocholic, chenodeoxycholic, glycochenodeoxycholic, or taurochenodeoxycholic acid. The bile acids and deconjugated bile acids were then extracted with nordeoxycholic acid, hydrochloric acid and ethyl acetate, and quantified by HPLC.

5. Biogenic amines

B. breve M-16V was incubated anaerobically in potassium phosphate buffer (30 mM, pH 5.0) with or without L-histidine (10 mM) or L-tyrosine (10 mM) for 5 h at 37°C. The pH of the buffer was then measured and compared to that of the mixture immediately after *B. breve* M-16 was added.

6. Ammonia production

Ammonia production was determined as described (Di Giorgio, 1974; Lin and Visek, 1991). One hundred µl of 30 % trichloric acid and 900 µl BHI broth harvested from cultures of *B. breve* M-16V, *L. rhamnosus*, or *Enterococcus faecium* grown anaerobically for 48 h at 37°C was incubated with 75 µl of solution containing 50 mg/ml phenol, 0.24 mg/ml sodium nitroprusside and 75 µl of alkali-hypochlorite for 20 min at 37 °C. One hundred µl of deionized water was then added to the mixture and the absorbance at 620 nM was measured.

7. Azoreductase activity

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Azoreductase activity was determined as described (Rafii et al., 1990). Bacteria were grown anaerobically on brain heart infusion (BHI) agar plates containing Direct Blue 15, Nitro Red, Sunset Yello FCF, and Amaranth food dye E213 for 72 h at 37°C. The plates were then analyzed for discoloration of the different dyes.

8. Nitroreductase activity

B. breve M-16V was cultured anaerobically in Sheadler medium for 48 h at 37°C. The medium was then harvested and incubated with para-nitrobenzoic acid and NADH/NADPH. The absorbance of the reduced product was determined at 540nm and compared to a standard curve generated using para-aminobenzoic acid.

9. Hemolytic potential

B. breve M-16V and *Bifidobacterium longum* BB536 were cultured anaerobically on GAM agar (Nissui Pharmaceutical Co., Ltd.) supplemented with sheep blood for 48 h at 37°C. *Listeria ivanovii* ATCC 19119 was cultured aerobically on GAM agar supplemented sheep blood for 24 h at 37°C. The plates were then analyzed for the presence of hemolytic zones surrounding the colonies of bifidobacteria and listeria.

10. Mucolytic activity

B. breve M-16V mucolytic activity was determined as described (Abe et al., 2010).

11. Mutagenicity

Agar medium containing *Salmonella typhimurium* TA100 or TA98 was inoculated with increasing amounts of suspended M-16V powder, furylfuramide (AF-2), or 2-aminoanthracene (2-AA) with or without the metabolic activation agent S9 and incubated for 72 hour at 37°C. The colonies were then counted and recorded.

B. In vivo safety studies

1. Oral toxicity

The oral toxicity of *B. breve* M-16V was determined as described (Abe et al., 2009b).

2. Intravenous toxicity

ICR mice (Charles River) were injected intraperitoneally with or without cyclophosphamide (250 mg/kg) and, two days later, injected intravenously with

Appendix 4

increasing amounts of bifidobacteria (2×10^6 to 5×10^{10} cfu). Bacteria-induced mortality rate was then monitored over 14 days.

3. Translocation activity

One hundred million cfu of bifidobacteria were administered orally to four to six week-old male ICR mice (Charles River) for five consecutive days. On the fourth, sixth, and eighth day after the initial dose of bifidobacteria, the mice were injected intraperitoneally with cyclophosphamide (day 4, 250 mg/kg; day 6, 250 mg/kg; and day 8, 150 mg/kg). On day 9 the mice were euthanized and organ homogenates were cultured aerobically and anaerobically on trypticase soy agar or on BL agar medium. The colonies were counted and evaluated microscopically 24 h later.

V. References

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APPENDIX 5A

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**MORINAGA MILK INDUSTRY CO., LTD.**

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Date: Feb 21, 2012

Certificate of Analysis

Product : M-16V (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2009.06.16

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	$> 1.0 \times 10^{11}$	2.3×10^{11}
Aerobic CFU	CFU/g	< 300	$< 300(0)$
Enterobacteriaceae	/0.01g	Negative	Negative
<i>Escherichia coli</i>	/g	Negative	Negative
Enterococci	/0.01g	Negative	Negative
<i>Clostridium perfringens</i>	/0.1g	Negative	Negative
<i>Staphylococcus aureus</i>	/0.01g	Negative	Negative
Mold	CFU/g	< 30	$< 10(0)$
Yeast	CFU/g	< 30	$< 10(0)$
<i>Salmonella</i>	/25g	Negative	Negative
<i>Cronobacter sakazaki</i>	/25g	Negative	Negative
<i>Bacillus cereus</i>	CFU/g	< 100	$< 30(0)$
Lead	ppm	< 0.2	< 0.2
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	0.6
Casein	ppm	< 0.1	< 0.1
β -Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co., Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

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Date:Feb 21,2012

Certificate of Analysis

Product : M-16V (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2009.10.28

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	> 1.0 x 10 ¹¹	2.3x 10 ¹¹
Aerobic CFU	CFU/g	<300	<300(0)
Enterobacteriaceae	/0.01g	Negative	Negative
Escherichia coli	/g	Negative	Negative
Enterococci	/0.01g	Negative	Negative
Clostridium perfringens	/ 0.1g	Negative	Negative
Staphylococcus aureus	/0.01g	Negative	Negative
Mold	CFU/g	<30	<10(0)
Yeast	CFU/g	<30	<10(0)
Salmonella	/25g	Negative	Negative
Cronobacter sakazaki	/25g	Negative	Negative
Bacillus cereus	CFU/g	<100	<30(0)
Lead	ppm	<0.2	<0.2
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	1.7
Casein	ppm	< 0.1	< 0.1
β-Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co.,Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

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Date: Feb 21, 2012

Certificate of Analysis

Product : M-16V (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2010.02.17

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	$> 1.0 \times 10^{11}$	2.0×10^{11}
Aerobic CFU	CFU/g	<300	<300(0)
Enterobacteriaceae	/0.01g	Negative	Negative
Escherichia coli	/g	Negative	Negative
Enterococci	/0.01g	Negative	Negative
Clostridium perfringens	/0.1g	Negative	Negative
Staphylococcus aureus	/0.01g	Negative	Negative
Mold	CFU/g	<30	<10(0)
Yeast	CFU/g	<30	<10(0)
Salmonella	/25g	Negative	Negative
Cronobacter sakazaki	/25g	Negative	Negative
Bacillus cereus	CFU/g	<100	<30(0)
Lead	ppm	<0.2	<0.2
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	1.9
Casein	ppm	< 0.1	< 0.1
β -Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co., Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

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Date: Feb 21, 2012

Certificate of Analysis

Product : M-16V (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2010.11.10

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	> 1.0 x 10 ¹¹	1.9x 10 ¹¹
Aerobic CFU	CFU/g	<300	<300(0)
Enterobacteriaceae	/0.01g	Negative	Negative
Escherichia coli	/g	Negative	Negative
Enterococci	/0.01g	Negative	Negative
Clostridium perfringens	/ 0.1g	Negative	Negative
Staphylococcus aureus	/0.01g	Negative	Negative
Mold	CFU/g	<30	<10(0)
Yeast	CFU/g	<30	<10(0)
Salmonella	/25g	Negative	Negative
Cronobacter sakazaki	/25g	Negative	Negative
Bacillus cereus	CFU/g	<100	<30(0)
Lead	ppm	<0.2	<0.2
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	1.2
Casein	ppm	< 0.1	< 0.1
β-Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co.,Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

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Date: Feb 21, 2012

Certificate of Analysis

Product : M-16V (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2011.03.01

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	$> 1.0 \times 10^{11}$	2.0×10^{11}
Aerobic CFU	CFU/g	< 300	$< 300(0)$
Enterobacteriaceae	/0.01g	Negative	Negative
<i>Escherichia coli</i>	/g	Negative	Negative
Enterococci	/0.01g	Negative	Negative
<i>Clostridium perfringens</i>	/0.1g	Negative	Negative
<i>Staphylococcus aureus</i>	/0.01g	Negative	Negative
Mold	CFU/g	< 30	$< 10(0)$
Yeast	CFU/g	< 30	$< 10(0)$
<i>Salmonella</i>	/25g	Negative	Negative
<i>Cronobacter sakazaki</i>	/25g	Negative	Negative
<i>Bacillus cereus</i>	CFU/g	< 100	$< 30(0)$
Lead	ppm	< 0.2	< 0.2
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	2.0
Casein	ppm	< 0.1	< 0.1
β -Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co., Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

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APPENDIX 5B

**MORINAGA MILK INDUSTRY CO., LTD.**

33-1, SHIBA 5-CHOME, MINATO-KU, TOKYO 108-8384, JAPAN

TEL : 81-3-3798-0152

FAX : 81-3-3798-0107

E-mail: interntl@morinagamilk.co.jp

Date: Feb 21, 2012

Certificate of Analysis

Product : M-16V type-T (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2006.01.31

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	$> 1.0 \times 10^{11}$	2.0×10^{11}
Aerobic CFU	CFU/g	< 300	$< 300(0)$
Coliform bacteria	/g	Negative	Negative
<i>Staphylococcus aureus</i>	/0.01g	Negative	Negative
Mold	CFU/g	< 30	$< 10(0)$
Yeast	CFU/g	< 30	$< 10(0)$
Salmonella	/5g	Negative	Negative
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	0.7
Casein	ppm	< 0.1	< 0.1
β -Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co., Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

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**MORINAGA MILK INDUSTRY CO., LTD.**

33-1, SHIBA 5-CHOME, MINATO-KU, TOKYO 108-8384, JAPAN

TEL : 81-3-3798-0152

FAX : 81-3-3798-0107

E-mail: internt@morinagamilk.co.jp

Date:Feb 21,2012

Certificate of Analysis

Product : M-16V type-T (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2006.04.28

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	> 1.0 x 10 ¹¹	2.1x 10 ¹¹
Aerobic CFU	CFU/g	<300	<300(0)
Coliform bacteria	/g	Negative	Negative
Staphylococcus aureus	/0.01g	Negative	Negative
Mold	CFU/g	<30	<10(0)
Yeast	CFU/g	<30	<10(0)
Salmonella	/5g	Negative	Negative
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	0.8
Casein	ppm	< 0.1	< 0.1
β -Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co.,Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

000185

**MORINAGA MILK INDUSTRY CO., LTD.**

33-1, SHIBA 5-CHOME, MINATO-KU, TOKYO 108-8384, JAPAN

TEL : 81-3-3798-0152

FAX : 81-3-3798-0107

E-mail: interntl@morinagamilk.co.jp

Date: Feb 21, 2012

Certificate of Analysis

Product : M-16V type-T (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2006.12.01

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	$> 1.0 \times 10^{11}$	2.1×10^{11}
Aerobic CFU	CFU/g	< 300	$< 300(0)$
Coliform bacteria	/g	Negative	Negative
<i>Staphylococcus aureus</i>	/0.01g	Negative	Negative
Mold	CFU/g	< 30	$< 10(0)$
Yeast	CFU/g	< 30	$< 10(0)$
Salmonella	/5g	Negative	Negative
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	1.0
Casein	ppm	< 0.1	< 0.1
β -Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co., Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

000186

**MORINAGA MILK INDUSTRY CO., LTD.**

33-1, SHIBA 5-CHOME, MINATO-KU, TOKYO 108-8384, JAPAN

TEL : 81-3-3798-0152

FAX : 81-3-3798-0107

E-mail: interntl@morinagamilk.co.jp

Date: Feb 21, 2012

Certificate of Analysis

Product : M-16V type-T (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2008.09.22

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	$> 1.0 \times 10^{11}$	1.8×10^{11}
Aerobic CFU	CFU/g	< 300	$< 300(0)$
Coliform bacteria	/g	Negative	Negative
Staphylococcus aureus	/0.01g	Negative	Negative
Mold	CFU/g	< 30	$< 10(0)$
Yeast	CFU/g	< 30	$< 10(0)$
Salmonella	/5g	Negative	Negative
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	1.0
Casein	ppm	< 0.1	< 0.1
β -Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co., Ltd.
Analytical Research Center

(b) (6)


Motonori Toyoda

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MORINAGA MILK INDUSTRY CO., LTD.

33-1, SHIBA 5-CHOME, MINATO-KU, TOKYO 108-8384, JAPAN

TEL : 81-3-3798-0152
FAX : 81-3-3798-0107
E-mail: interntl@morinagamilk.co.jp

Date: Feb 21, 2012

Certificate of Analysis

Product : M-16V type-T (*Bifidobacterium breve* M-16V)
MANUFACTURING DATE : 2011.06.21

Parameter	Unit	Specification	Analysis Data
Appearance		White to slightly brown powder	Passed
Foreign body		Negative	Passed
Odor and Taste		No abnormal odor and taste	Passed
Anaerobic CFU (<i>B. breve</i> M-16V)	CFU/g	$> 1.0 \times 10^{11}$	2.5×10^{11}
Aerobic CFU	CFU/g	<300	<300(0)
Coliform bacteria	/g	Negative	Negative
<i>Staphylococcus aureus</i>	/0.01g	Negative	Negative
Mold	CFU/g	<30	<10(0)
Yeast	CFU/g	<30	<10(0)
<i>Salmonella</i>	/5g	Negative	Negative
Arsenic	ppm	< 1	< 1
Other heavy Metals	ppm	< 5	< 5
Loss on Drying	g/100g	< 6	0.7
Casein	ppm	< 0.1	< 0.1
β -Lactoglobulin	ppm	< 0.1	< 0.1

Conclusion

To pass the standard

Morinaga Milk Industry Co., Ltd.
Analytical Research Center

(b) (6)

Motonori Toyoda

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BLASTN Analysis for Antibiotic Resistance Genes in the <i>B. breve</i> M-16V Genomic Sequence ¹							
Gene ID Number	Gene Accession Number	Gene symbol	Definition	Source Organism	Length (bp)	Score	E-value
154485921	NZ_AAXD02000001.1	cata 1	hypothetical protein BIFADO_00004	<i>Bifidobacterium adolescentis</i> L2-32	660	34.2	0.075
224284016	NZ_ABQP01000028	tetA	tetracycline resistance structural protein TetA	<i>Bifidobacterium bifidum</i>	1191	36.2	0.15
67005923	AAV62597.1	tetW	tetracycline resistance gene	<i>Bifidobacterium longum</i>	1924	34.2	0.22
168259016	ACA23195.1	tetW	tetracycline resistance gene	<i>Bifidobacterium bifidum</i>	1950	34.2	0.22
215490332	FJ441296.1	aadE	aminoglycoside adenylyltransferase-like gene	<i>Enterococcus faecium</i>	575	32.2	0.26
125662885	DQ988362.1	tetW	tetracycline resistance gene	<i>Bifidobacterium lactis</i>	709	32.2	0.32
125662865	DQ988352.1	tetW	tetracycline resistance gene	<i>Bifidobacterium breve</i> strain 55	722	32.2	0.32
125662873	DQ988356.1	tetW	tetracycline resistance gene	<i>Bifidobacterium bifidus</i> strain 163	723	32.2	0.33
14456347	AAK62562.1	aphA3	aminoglycoside phosphotransferase	<i>Enterococcus faecium</i>	800	32.2	0.36
22652807	AF503772.1	tetL	TetL transporter	<i>Enterococcus faecalis</i> DS5 plasmid pAMalpha1	819	32.2	0.37
188593359	AM748803.1	erm(X)	erythromycin resistance gene	<i>Bifidobacterium animalis</i> transposon Tn5432, strain B0456	898	32.2	0.4
14456345	AAK62560.1	aad(6)	aminoglycoside adenylyltransferase	<i>Enterococcus faecium</i>	920	32.2	0.41
188593354	AM748801.1	erm(X)	erythromycin resistance gene	<i>Bifidobacterium thermophilum</i> transposon Tn5432, strain B0225	1338	32.3	0.6
73665546	AAZ79478.1	tetQ	tetracycline resistance gene	<i>Bifidobacterium bifidum</i> plasmid	1980	32.2	0.87
56155284	AY660532.1	tetO	tetracycline resistance gene	<i>Enterococcus faecalis</i> strain e291	1947	32.2	0.88
28273038	AJ488494.2	vatE	streptogramin A resistance protein	<i>Lactobacillus fermentum</i> plasmid pLME300	657	30.2	1.15
42494908	AAS17731.1	tetC	tetracycline resistance gene	<i>Escherichia coli</i> transposon Tn10	680	30.2	1.2
110556094	AB247327.1	ermB	erythromycin resistance gene	<i>Enterococcus faecalis</i>	760	30.2	1.34
55709829	AAV58814.1	vanWB		<i>Clostridium</i> sp CCRI-9842 transposon Tn5382	840	30.2	1.49
1667471	U75299.1	cat-TC	chloramphenicol acetyltransferase-TC gene	<i>Lactobacillus reuteri</i> plasmid pTC82	850	30.2	1.5
209693322	ACD88490.1	mefA	macrolide-efflux protein A	<i>Clostridium perfringens</i>	1220	30.2	2.17
28373205	NP_783842.1	vatE	streptogramin A resistance protein	<i>Lactobacillus fermentum</i> ROT1 plasmid	670	30.2	2.27

BLASTN Analysis for Antibiotic Resistance Genes in the <i>B. breve</i> M-16V Genomic Sequence ¹							
GenInfo (GI) Number	Gene Accession Number	Gene symbol	Definition	Source Organism	Length (bp)	Score	E-value ²
				pLME300			
84663560	DQ304773.1	mefA	macrolide-efflux protein gene	<i>Streptococcus mitis</i>	1344	30.2	2.4
1835780	U86375.1	ermB	erythromycin resistance gene	<i>Enterococcus faecalis</i>	1482	30.2	2.65
118135710	EF070727.1	ermB	erythromycin resistance gene	<i>Lactobacillus salivarius</i> strain BFE 7441	1483	28.2	2.8
121999251	NC_008790.1	tetO	tetracycline resistance gene	<i>Canpylobacter jejuni</i>	1920	30.2	3.2
146188919	CAJ21496.1	tetM	tetracycline resistance gene	<i>Clostridium difficile</i>	1930	30.2	3.45
15127841	AF368302.1	vatD ³	streptogramin A acetyltransferase	<i>Enerococcus faecium</i>	615	28.2	4.38
115349861	DQ910316.1	tetS	tetracycline resistance gene	<i>Lactococcus</i> sp. LP-P1-2	627	28.2	4.4
220897973	FJ489650.1	ermC ⁴	erythromycin resistance protein	<i>Lactobacillus reuteri</i> strain PA-16 plasmid	740	28.2	5.16
300910321	NZ_ACGW02000014.1	lnuA	lincosamide nucleotidyltransferase	<i>Lactobacillus reuteri</i> SD2112	490	28.2	6.36

¹The genome of *B. breve* M-16V was searched for regions with homology to antibiotic resistance genes found in lactic acid-producing bacteria.

²E-value of 0 to 1E-100 equals high homology; E-value of 1E-100 to 1E-50 equals medium homology; E-value of 1E-50 to 1E-30 equals low homology; E-values of >1E-30 equals little to homology. All genes queried fell above an E-value of 1E-30, indicating there is very little homology between its sequence and the genomic sequence of the *B. breve* M-16V.

³The 615 bp used in the BLASTN analysis of GI:15127841 (vatD) correspond to the nucleotides found between 2717 and 3331 of its cDNA.

⁴The 740 bp used for BLASTN analysis of GI:220897973 (ermC) correspond to the nucleotides found between 2376 and 3116

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APPENDIX 7A

000191



FACULTY OF SCIENCES

Department of **Biochemistry, Physiology & Microbiology**

Laboratory of **Microbiology**

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your reference

contact
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our reference

e-mail
Geert.Huys@UGent.be

date

19-09-2007

phone and fax

T +32 9 264 51 31
F +32 9 264 50 92

Beste Richèle,

Gelieve in bijlage de tweede versie van het **finale rapport** van het service contract rond antimicrobiële gevoeligheidsbepalingen van NUMICO culturen te vinden. Het rapport werd in het Engels opgemaakt omdat dit de taal is die standaard wordt gebruikt bij rapportering van resultaten voor buitenlandse klanten. Als Annex aan dit rapport werden twee tabellen (Table 1-2) toegevoegd.

Aarzel niet om mij te contacteren voor verdere informatie.

Met vriendelijke groeten,

Geert Huys
Contact persoon

000192

FINAL REPORT service contract NUMICO

'Antimicrobial susceptibility determinations of NUMICO cultures'

1. Short description of the study

The Services that were provided by UGent include antibiotic susceptibility testing of 12 strains of lactic acid bacteria for six antibiotics. Susceptibility testing was conducted using the **broth dilution method** according to the SOP **ACE-ART-MDIL** that have been approved by the partners of the ACE-ART project (<http://www.aceart.net/>). The description of this method has meanwhile been revised by the Joint Action Team on Probiotics of the International Dairy Federation resulting in a tentative ISO standard (ISO TC 34/SC 5N) which is currently under evaluation for assessment of inter-laboratory performance. The test medium is based on the LSM formulation described by Klare and co-workers:

Klare, I., C. Konstabel, S. Müller-Bertling, R. Reissbrodt, G. Huys, M. Vancanneyt, J. Swings, H. Goossens & W. Witte. 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Applied and Environmental Microbiology 71:8982-8986.

The **broth dilution method** is based on the inoculation of a standardized broth inoculum of the test strain in a dilution series of the antibiotic for which the MIC is determined. The first concentration in the dilution series at which no visual growth can be determined was considered as the MIC. The following antibiotics were tested using premade **VetMIC™ ACE-ART microdilution plates**:

Antibiotic	Class	Concentration range
Tetracycline	Tetracyclines	0.5 - 128 µg/ml
Erythromycin	Macrolides	0.12 - 16 µg/ml
Streptomycin	Aminoglycosides	2 - 256 µg/ml
Gentamicin	Aminoglycosides	0.5 - 32 µg/ml
Clindamycin	Lincosamides	0.12 - 8 µg/ml
Ampicillin	Penicillins	0.12 - 8 µg/ml

All tests were performed in duplicate during independent assays starting from new bacterial subcultures. MICs were read after **48h incubation**.

2. Results of MIC measurements

MIC measurements obtained with VetMIC were compiled in Table 1 attached as Annex to this report. This table lists the MIC results of six agents for each of the 12 cultures in mg/L.

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3. Discussion

3.1. Technical performance

- In general, the standard deviation between a series of repeated MIC determinations is usually one log₂ step. Taking this into consideration, a good to excellent correspondence was observed between replicate results for each strain-antibiotic test combination (Table 1).

- In each test assay, a control strain was included i.e. *Lactobacillus plantarum* ATCC 14917^T (= LMG 6907^T) for tests read at 28°C and *Enterococcus faecalis* ATCC 29212 (= LMG 8222) for tests read at 37°C (Table 2). Inter-assay MIC measurements for these two control strains were in good agreement (0-1 log₂ variation).

3.2. Identification of strains with atypical phenotypic resistance traits

At present, no widely accepted interpretive guidelines have been proposed for antimicrobial susceptibility testing of non-enterococcal lactic acid bacteria. Following the EUCAST definition (<http://www.eucast.org>), an epidemiological cut-off value serves to distinguish between **wild type** organisms (free of acquired and mutational resistance mechanisms to the tested agent) and **non-wild type** organisms (harbouring an acquired or mutational resistance mechanism to the tested agent). In the present study, the interpretation given below strongly relies on the results and tentative guidelines of two ongoing EU research projects that have dedicated work packages in this field i.e. **EU-PROSAFE** (2002-2006; coordinator: Prof. Herman Goossens, University of Antwerp, Belgium; e-mail: Herman.Goossens@uza.be) and **EU-ACE-ART** (2004-2006; coordinator: Prof. Lorenzo Morelli, University of Piacenza, Italy; e-mail: Lorenzo.Morelli@unicatt.it).

By comparing with tentative guidelines based on PROSAFE and ACE-ART data or literature data (whenever available for a particular species), **two strains** of the panel of 12 investigated cultures were found to contain potentially atypical resistance traits. For the other 10 cultures tested, susceptibility data did not reveal the presence of such traits. In Table 1, MIC measurements that are atypical within the given LAB species and that indicate that a specific strain belongs to the non-wild type population within that species are indicated in pink. MIC data potentially indicating resistance but awaiting further confirmation are in green.

***Bifidobacterium breve* 247**

For **tetracycline** (Tc), an MIC of 32 mg/L (VetMIC) was recorded for strain **247** whereas four other tested strains of this species, i.e. 200, 204, 226 and 239, displayed MICs for Tc in the range 1-2 mg/L. Based on the tentative PROSAFE guideline, a cut-off of ≤8 mg/L Tc is proposed to define wild-type strains in this species. In the recent study of Masco and co-workers (*L. Masco, K. Van Hoorde, E. De Brandt, J. Swings & G. Huys. 2006. Antimicrobial susceptibility of Bifidobacterium strains from humans, animals and probiotic products. Journal of Antimicrobial Chemotherapy 58:85-94*), it was reported that *Bifidobacterium* strains exhibiting MICs for Tc ≥4 mg/L contain the ribosomal protection gene *tet(W)*, but also *tet(M)* and *tet(O)* have been reported in *Bifidobacterium* strains (*J. Aires, F. Doucet-Populaire & M.J. Butel. 2007. Tetracycline resistance mediated by tet(W), tet(M), and tet(O) genes of Bifidobacterium isolates from humans. Applied and Environmental Microbiology 73:2751-2754*). **From these data, it can be concluded that strain 247 probably exhibits acquired atypical resistance (probably due an acquired *tet* gene) to Tc.**

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***Bifidobacterium breve* 253**

For Tc, an MIC of 16 mg/L (VetMIC) was recorded for strain **253** whereas four other tested strains of this species, i.e. 200, 204, 226 and 239, displayed MICs for Tc in the range 1-2 mg/L. Based on the tentative PROSAFE guideline, a cut-off of ≤ 8 mg/L Tc is proposed to define wild-type strains in this species. In the recent study of Masco and co-workers (*Masco et al., 2006*), it was reported that *Bifidobacterium* strains exhibiting MICs for Tc ≥ 4 mg/L contain the ribosomal protection gene *tet(W)*, but also *tet(M)* and *tet(O)* have been reported in *Bifidobacterium* strains (*Aires et al., 2007*).

For **streptomycin** (Sm), an MIC of >256 mg/L (VetMIC) was recorded for strain **253** whereas five other tested strains of this species, i.e. 200, 204, 226, 239 and 247 displayed MICs for Sm in the range 32-128 mg/L. Lactic acid bacteria and bifidobacteria display intrinsic, medium-level resistance to aminoglycosides such as Sm due to the lack of a cytochrome-mediated drug transport (*Masco et al., 2006*). However, an MIC of >256 mg/L may point to the presence of high-level Sm resistance in strain 253 mediated by mutation or gene acquisition, but further determination of the MIC end-point is required to substantiate this conclusion.

From these data, it can be concluded that strain 253 probably exhibits acquired atypical resistance (probably due an acquired *tet* gene) to Tc. Extension of the MIC dilution series is required to decide on the possible presence of high-level Sm resistance.

4. Conclusions

In this study, MICs of six antimicrobial agents were determined for a panel of 12 LAB cultures using the recently described LSM medium and VetMIC methodology.

Overall, MIC data were reproducible and allowed to identify strains with atypical resistance traits that may correspond to the non-wild type population within a given species. Following comparison with other data obtained in this study, with data from the PROSAFE and ACE-ART projects and from literature, it can be concluded that 10 out of the 12 strains did not display phenotypic resistance to any of the six tested agents. In contrast, the following **two strains** most probably exhibit **acquired atypical resistance**:

<i>Bifidobacterium breve</i> 247:	resistance to Tc
<i>Bifidobacterium breve</i> 253:	resistance to Tc possible high-level resistance to Sm

Depending on the industrial use of the two strains identified with a high probability of acquired resistance, further analysis of these strains is recommended to determine the **genetic basis** of the observed resistances including PCR-based detection of *tet* resistance (and other) genes commonly found in Gram-positives, localization of these genes (if present!) on the chromosome or on mobile genetic elements and initiation of horizontal transferability studies.

Geert Huys
September 19, 2007

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APPENDIX 7B

000196

Table 1. Results of MIC testing of 12 LAB cultures

Original no	Taxon received as	Medium	Agent	MIC (VetMIC)	Incubation t	Incubation T	Atmosphere	Date
200	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	15-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	21-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	15-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	21-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	128	48u	37°C	anaerobic	15-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	64	48u	37°C	anaerobic	21-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	15-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	21-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	>32	48u	37°C	anaerobic	15-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	>32	48u	37°C	anaerobic	21-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	0,25	48u	37°C	anaerobic	15-6-2007
200	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	0,25	48u	37°C	anaerobic	21-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	32	48u	37°C	anaerobic	15-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	32	48u	37°C	anaerobic	21-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	15-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	<0,12	48u	37°C	anaerobic	21-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	32	48u	37°C	anaerobic	15-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	32	48u	37°C	anaerobic	21-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	15-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	21-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	32	48u	37°C	anaerobic	15-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	32	48u	37°C	anaerobic	21-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	2	48u	37°C	anaerobic	15-6-2007
247	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	2	48u	37°C	anaerobic	21-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	1	48u	37°C	anaerobic	15-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	21-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	15-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	21-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	32	48u	37°C	anaerobic	15-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	32	48u	37°C	anaerobic	21-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	15-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	21-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	16	48u	37°C	anaerobic	15-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	32	48u	37°C	anaerobic	21-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	2	48u	37°C	anaerobic	15-6-2007
226	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	1	48u	37°C	anaerobic	21-6-2007

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Table 1. Results of MIC testing of 12 LAB cultures

204	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	15-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	21-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	15-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	<0,12	48u	37°C	anaerobic	21-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	32	48u	37°C	anaerobic	15-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	32	48u	37°C	anaerobic	21-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,50	48u	37°C	anaerobic	15-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	21-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	8	48u	37°C	anaerobic	15-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	16	48u	37°C	anaerobic	21-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	2	48u	37°C	anaerobic	15-6-2007
204	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	1	48u	37°C	anaerobic	21-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	15-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	21-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	15-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	<0,12	48u	37°C	anaerobic	21-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	32	48u	37°C	anaerobic	15-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Streptomycin	64	48u	37°C	anaerobic	21-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,50	48u	37°C	anaerobic	15-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	21-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	32	48u	37°C	anaerobic	15-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	16	48u	37°C	anaerobic	21-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	2	48u	37°C	anaerobic	15-6-2007
239	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	1	48u	37°C	anaerobic	21-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	16	48u	37°C	anaerobic	15-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Tetracycline	16	48u	37°C	anaerobic	21-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	<0,12	48u	37°C	anaerobic	15-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	21-6-2007
253	<i>B. breve</i>	LSM + L-cysteine			48u	37°C	anaerobic	15-6-2007
253	<i>B. breve</i>	LSM + L-cysteine			48u	37°C	anaerobic	21-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,50	48u	37°C	anaerobic	15-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	21-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	16	48u	37°C	anaerobic	15-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Gentamicin	32	48u	37°C	anaerobic	21-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	2	48u	37°C	anaerobic	15-6-2007
253	<i>B. breve</i>	LSM + L-cysteine	Ampicillin	1	48u	37°C	anaerobic	21-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	15-6-2007

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Table 1. Results of MIC testing of 12 LAB cultures

9	<i>B. longum</i>	LSM + L-cysteine	Tetracycline	2	48u	37°C	anaerobic	21-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	15-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Clindamycin	0,25	48u	37°C	anaerobic	21-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Streptomycin	64	48u	37°C	anaerobic	15-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Streptomycin	64	48u	37°C	anaerobic	21-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Erythromycin	0,50	48u	37°C	anaerobic	15-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	21-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Gentamicin	>32	48u	37°C	anaerobic	15-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Gentamicin	32	48u	37°C	anaerobic	21-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Ampicillin	1	48u	37°C	anaerobic	15-6-2007
9	<i>B. longum</i>	LSM + L-cysteine	Ampicillin	1	48u	37°C	anaerobic	21-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Tetracycline	1	48u	37°C	anaerobic	15-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Tetracycline	1	48u	37°C	anaerobic	21-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Clindamycin	<0,12	48u	37°C	anaerobic	15-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Clindamycin	<0,12	48u	37°C	anaerobic	21-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Streptomycin	128	48u	37°C	anaerobic	15-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Streptomycin	64	48u	37°C	anaerobic	21-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Erythromycin	0,25	48u	37°C	anaerobic	15-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Erythromycin	0,50	48u	37°C	anaerobic	21-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Gentamicin	>32	48u	37°C	anaerobic	15-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Gentamicin	32	48u	37°C	anaerobic	21-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Ampicillin	1	48u	37°C	anaerobic	15-6-2007
252	<i>B. longum</i>	LSM + L-cysteine	Ampicillin	1	48u	37°C	anaerobic	21-6-2007
11	<i>L. paracasei</i>	LSM	Tetracycline	1	48u	28°C	anaerobic	15-6-2007
11	<i>L. paracasei</i>	LSM	Tetracycline	1	48u	28°C	anaerobic	21-6-2007
11	<i>L. paracasei</i>	LSM	Clindamycin	0,50	48u	28°C	anaerobic	15-6-2007
11	<i>L. paracasei</i>	LSM	Clindamycin	0,50	48u	28°C	anaerobic	21-6-2007
11	<i>L. paracasei</i>	LSM	Streptomycin	128	48u	28°C	anaerobic	15-6-2007
11	<i>L. paracasei</i>	LSM	Streptomycin	128	48u	28°C	anaerobic	21-6-2007
11	<i>L. paracasei</i>	LSM	Erythromycin	0,50	48u	28°C	anaerobic	15-6-2007
11	<i>L. paracasei</i>	LSM	Erythromycin	0,50	48u	28°C	anaerobic	21-6-2007
11	<i>L. paracasei</i>	LSM	Gentamicin	16	48u	28°C	anaerobic	15-6-2007
11	<i>L. paracasei</i>	LSM	Gentamicin	16	48u	28°C	anaerobic	21-6-2007
11	<i>L. paracasei</i>	LSM	Ampicillin	1	48u	28°C	anaerobic	15-6-2007
11	<i>L. paracasei</i>	LSM	Ampicillin	2	48u	28°C	anaerobic	21-6-2007
8	<i>L. plantarum</i>	LSM	Tetracycline	32	48u	28°C	anaerobic	15-6-2007
8	<i>L. plantarum</i>	LSM	Tetracycline	32	48u	28°C	anaerobic	21-6-2007

Table 1. Results of MIC testing of 12 LAB cultures

8	<i>L. plantarum</i>	LSM	Clindamycin	2	48u	28°C	anaerobic	15-6-2007
8	<i>L. plantarum</i>	LSM	Clindamycin	2	48u	28°C	anaerobic	21-6-2007
8	<i>L. plantarum</i>	LSM	Streptomycin	128	48u	28°C	anaerobic	15-6-2007
8	<i>L. plantarum</i>	LSM	Streptomycin	128	48u	28°C	anaerobic	21-6-2007
8	<i>L. plantarum</i>	LSM	Erythromycin	1	48u	28°C	anaerobic	15-6-2007
8	<i>L. plantarum</i>	LSM	Erythromycin	1	48u	28°C	anaerobic	21-6-2007
8	<i>L. plantarum</i>	LSM	Gentamicin	8	48u	28°C	anaerobic	15-6-2007
8	<i>L. plantarum</i>	LSM	Gentamicin	8	48u	28°C	anaerobic	21-6-2007
8	<i>L. plantarum</i>	LSM	Ampicillin	0,25	48u	28°C	anaerobic	15-6-2007
8	<i>L. plantarum</i>	LSM	Ampicillin	0,25	48u	28°C	anaerobic	21-6-2007
1	<i>L.rhamnosus</i>	LSM	Tetracycline	1	48u	37°C	anaerobic	15-6-2007
1	<i>L.rhamnosus</i>	LSM	Tetracycline	2	48u	37°C	anaerobic	21-6-2007
1	<i>L.rhamnosus</i>	LSM	Clindamycin	2	48u	37°C	anaerobic	15-6-2007
1	<i>L.rhamnosus</i>	LSM	Clindamycin	2	48u	37°C	anaerobic	21-6-2007
1	<i>L.rhamnosus</i>	LSM	Streptomycin	16	48u	37°C	anaerobic	15-6-2007
1	<i>L.rhamnosus</i>	LSM	Streptomycin	16	48u	37°C	anaerobic	21-6-2007
1	<i>L.rhamnosus</i>	LSM	Erythromycin	0,50	48u	37°C	anaerobic	15-6-2007
1	<i>L.rhamnosus</i>	LSM	Erythromycin	0,50	48u	37°C	anaerobic	21-6-2007
1	<i>L.rhamnosus</i>	LSM	Gentamicin	8	48u	37°C	anaerobic	15-6-2007
1	<i>L.rhamnosus</i>	LSM	Gentamicin	8	48u	37°C	anaerobic	21-6-2007
1	<i>L.rhamnosus</i>	LSM	Ampicillin	1	48u	37°C	anaerobic	15-6-2007
1	<i>L.rhamnosus</i>	LSM	Ampicillin	2	48u	37°C	anaerobic	21-6-2007
6	<i>L.rhamnosus</i>	LSM	Tetracycline	1	48u	37°C	anaerobic	15-6-2007
6	<i>L.rhamnosus</i>	LSM	Tetracycline	1	48u	37°C	anaerobic	21-6-2007
6	<i>L.rhamnosus</i>	LSM	Clindamycin	2	48u	37°C	anaerobic	15-6-2007
6	<i>L.rhamnosus</i>	LSM	Clindamycin	2	48u	37°C	anaerobic	21-6-2007
6	<i>L.rhamnosus</i>	LSM	Streptomycin	16	48u	37°C	anaerobic	15-6-2007
6	<i>L.rhamnosus</i>	LSM	Streptomycin	16	48u	37°C	anaerobic	21-6-2007
6	<i>L.rhamnosus</i>	LSM	Erythromycin	0,50	48u	37°C	anaerobic	15-6-2007
6	<i>L.rhamnosus</i>	LSM	Erythromycin	0,50	48u	37°C	anaerobic	21-6-2007
6	<i>L.rhamnosus</i>	LSM	Gentamicin	4	48u	37°C	anaerobic	15-6-2007
6	<i>L.rhamnosus</i>	LSM	Gentamicin	4	48u	37°C	anaerobic	21-6-2007
6	<i>L.rhamnosus</i>	LSM	Ampicillin	1	48u	37°C	anaerobic	15-6-2007
6	<i>L.rhamnosus</i>	LSM	Ampicillin	1	48u	37°C	anaerobic	21-6-2007

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APPENDIX 7C

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Table 2. Results of MIC testing of LMG control strains

Original no	Taxon	Medium	Agent	MIC (VetMIC)	Incubation t	Incubation T	Atmosphere	Date
LMG 6907	<i>L. plantarum</i>	LSM	Tetracycline	32	48u	28°C	anaerobic	15-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Tetracycline	64	48u	28°C	anaerobic	21-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Clindamycin	2	48u	28°C	anaerobic	15-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Clindamycin	2	48u	28°C	anaerobic	21-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Streptomycin	256	48u	28°C	anaerobic	15-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Streptomycin	256	48u	28°C	anaerobic	21-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Erythromycin	1	48u	28°C	anaerobic	15-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Erythromycin	2	48u	28°C	anaerobic	21-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Gentamicin	8	48u	28°C	anaerobic	15-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Gentamicin	8	48u	28°C	anaerobic	21-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Ampicillin	0,50	48u	28°C	anaerobic	15-6-2007
LMG 6907	<i>L. plantarum</i>	LSM	Ampicillin	0,50	48u	28°C	anaerobic	21-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Tetracycline	8	48u	37°C	anaerobic	7-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Tetracycline	16	48u	37°C	anaerobic	15-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Tetracycline	16	48u	37°C	anaerobic	21-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Clindamycin	>8	48u	37°C	anaerobic	7-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Clindamycin	>8	48u	37°C	anaerobic	15-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Clindamycin	>8	48u	37°C	anaerobic	21-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Streptomycin	>256	48u	37°C	anaerobic	7-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Streptomycin	>256	48u	37°C	anaerobic	15-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Streptomycin	256	48u	37°C	anaerobic	21-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Erythromycin	4	48u	37°C	anaerobic	7-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Erythromycin	8	48u	37°C	anaerobic	15-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Erythromycin	8	48u	37°C	anaerobic	21-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Gentamicin	>32	48u	37°C	anaerobic	7-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Gentamicin	>32	48u	37°C	anaerobic	15-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Gentamicin	>32	48u	37°C	anaerobic	21-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Ampicillin	0,50	48u	37°C	anaerobic	7-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Ampicillin	0,50	48u	37°C	anaerobic	15-6-2007
LMG 8222	<i>E. faecalis</i>	LSM	Ampicillin	0,50	48u	37°C	anaerobic	21-6-2007

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BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query					Subject	Analysis											
GenBank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos	Gaps	HSP Length	Score	E-value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
AAG07658.1	PA4270	rpoB	DNA-directed RNA polymerase beta chain	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0428	1357	350	951	1187	252	862	54	70	23	618	664	0
BAB41321.1	SA0102		myosin-cross reactive MHC classII-like protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1344	591	1	591	625	1	625	53	71	38	627	702	0
BAB41731.1	SA0500	rpoB	RNA polymerase beta chain	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0428	1183	379	1162	1187	331	1157	60	73	63	837	977	0
BAB80084.1	CPE0378		probable myosin-crossreactive antigen	<i>Clostridium perfringens</i> str. 13	bbr01g1344	597	13	597	625	4	625	50	66	49	628	626	0
BAB82119.1		rpoB	RNA polymerase beta subunit	<i>Clostridium perfringens</i> str. 13	bbr01g0428	1234	423	1203	1187	369	1186	61	74	49	824	1011	0
AAL32278.1	U84782		signal recognition particle protein subunit	<i>Arcanobacterium pyogenes</i>	bbr01g0330	513	2	431	551	4	447	66	80	14	444	596	1.00E-172
AAL32277.1	U84782		signal recognition particle receptor	<i>Arcanobacterium pyogenes</i>	bbr01g0203	397	67	397	424	99	424	63	78	7	332	389	1.00E-110
AAL32275.1	U84782		chromosome segregation protein	<i>Arcanobacterium pyogenes</i>	bbr01g1231	268	6	262	1215	924	1213	53	66	33	290	286	6.00E-79
BAB80431.1		nanI	exo-alpha-sialidase	<i>Clostridium perfringens</i> str. 13	bbr01g0153	694	216	671	763	285	739	33	51	43	477	234	6.00E-63
BAB81588.1	CPE1882		probable collagenase	<i>Clostridium perfringens</i> str. 13	bbr01g1703	786	5	265	537	32	302	39	60	10	271	219	3.00E-58
BAB80259.1		nanJ	exo-alpha-sialidase	<i>Clostridium perfringens</i> str. 13	bbr01g0153	1173	378	820	763	311	755	31	47	46	467	212	6.00E-56

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query					Subject	Analysis											
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Ids	Pos	Gaps	HSP Length	Score	E-value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB41593.1	SA0366	ahpC	alkyl hydroperoxide reductase subunit C	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0024	189	1	189	187	1	187	46	70	2	189	201	1.00E-53
BAB80143.1		hlyB	probable hemolysin	<i>Clostridium perfringens</i> str. 13	bbr01g0019	445	31	429	421	1	400	28	54	35	417	203	1.00E-53
AAG03529.1	PA0139	ahpC	alkyl hydroperoxide reductase subunit C	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0024	187	1	187	187	1	187	43	66	0	187	192	7.00E-51
AAG07656.1	PA4268	rpsL	30S ribosomal protein S12	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0533	123	1	123	123	1	123	75	83	0	123	186	2.00E-49
BAB82116.1		rpsL	30S ribosomal protein S12	<i>Clostridium perfringens</i> str. 13	bbr01g0533	126	1	123	123	1	123	76	83	0	123	186	2.00E-49
BAB79736.1		hlyA	probable hemolysin-related protein	<i>Clostridium perfringens</i> str. 13	bbr01g0019	421	28	411	421	1	400	29	54	30	407	186	1.00E-48
BAB41734.1	SA0503	rpsL	30S ribosomal protein S12	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0533	137	1	136	123	1	123	71	76	13	136	183	2.00E-48
BAB41890.1	SA0657		probable hemolysin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0019	449	29	423	421	1	395	25	53	14	402	173	1.00E-44
BAB81524.1		hlyD	probable hemolysin	<i>Clostridium perfringens</i> str. 13	bbr01g0647	271	9	245	248	4	244	39	58	4	241	167	4.00E-43
BAB41500.1	SA0276		diarrheal toxin-like protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0681	1479	629	870	634	162	390	38	56	17	244	164	2.00E-41
BAB42019.1	SA0780		probable hemolysin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0019	346	38	322	421	16	312	30	50	30	306	124	4.00E-30
BAB41376.1	SA0156	capM	capsular polysaccharide synthesis enzyme Cap5M	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0378	185	1	184	469	274	469	34	51	14	197	119	5.00E-29

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BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query			Subject		Analysis												
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos	Gaps	HSP Lengths	Score	E-value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB79853.1		bglR	beta-glucuronidase	<i>Clostridium perfringens</i> str. 13	bbr01g0011	599	69	412	1040	143	480	27	39	56	369	119	3.00E-28
BAB41347.1	SA0127		probable capsular polysaccharide synthesis protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0385	476	2	397	486	9	406	26	49	12	403	114	6.00E-27
BAB41365.1	SA0145	capB	capsular polysaccharide synthesis enzyme Cap5B	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0403	228	13	211	478	238	439	31	53	3	202	110	4.00E-26
BAB79931.1		fhuC	ferrichrome ABC transporter	<i>Clostridium perfringens</i> str. 13	bbr01g0744	259	9	221	400	15	230	33	56	11	220	110	6.00E-26
BAB80519.1		fhuC	probable ferrichrome ABC transporter	<i>Clostridium perfringens</i> str. 13	bbr01g1688	260	4	209	259	5	217	33	54	15	217	107	4.00E-25
BAB41256.1	SA0038	mecA	penicillin binding protein 2 prime	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0057	668	258	636	488	68	466	25	43	54	416	101	1.00E-22
BAB81180.1		hlyC	probable hemolysin	<i>Clostridium perfringens</i> str. 13	bbr01g0678	215	10	211	208	1	200	34	49	8	205	85.1	2.00E-18
BAB81864.1	CPE215 8		probable adhesin	<i>Clostridium perfringens</i> str. 13	bbr01g0695	298	4	228	390	10	239	28	45	27	241	84.3	4.00E-18
BAB80013.1	CPE030 7		probable lipase	<i>Clostridium perfringens</i> str. 13	bbr01g0901	334	65	309	319	44	300	24	45	28	265	81.6	3.00E-17
BAB43766.1	SA2461	icaB	intercellular adhesion protein B	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1732	290	77	271	250	55	236	25	45	23	200	73.2	1.00E-14
BAB41266.1	SA0048	ermA	rRNA methylase	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1920	243	10	179	309	34	220	24	45	17	187	61.6	2.00E-11
BAB80448.1		lipB	probable lipase	<i>Clostridium perfringens</i> str. 13	bbr01g0524	573	331	567	371	78	341	24	40	55	278	63.9	2.00E-11

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BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query			Subject			Analysis											
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos	Gaps	HSP Length	Score	E-value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB41364.1	SA0144	capA	capsular polysaccharide synthesis enzyme Cap5A	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0403	222	38	218	478	33	225	24	44	12	193	60.5	4.00E-11
BAB43262.1	SA1973		probable hemolysin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0678	228	35	224	208	6	199	28	50	16	200	58.9	1.00E-10
BAB43764.1	SA2459	icaA	intercellular adhesion protein A	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0201	412	49	143	344	8	104	32	56	2	97	57.8	6.00E-10
BAB81621.1		hlyE	probable hemolysin III	<i>Clostridium perfringens</i> str. 13	bbr01g0077	213	49	211	217	42	212	27	45	14	174	55.5	2.00E-09
BAB41819.1	SA0587		probable adhesin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0695	309	11	160	390	20	159	24	43	26	158	54.7	4.00E-09
AAG06337.1	PA2949		probable lipase	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0831	315	58	294	278	17	259	22	44	14	247	54.3	6.00E-09
BAB41843.1	SA0610		probable lipase	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0901	347	53	299	319	42	302	20	39	46	277	53.5	9.00E-09
BAB41371.1	SA0151	capH	capsular polysaccharide synthesis enzyme O-acetyl transferase Cap5H	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0559	208	122	172	204	130	180	50	66	0	51	51.2	3.00E-08
BAB81060.1		entB	probable enterotoxin	<i>Clostridium perfringens</i> str. 13	bbr01g1564	549	463	549	251	165	251	41	57	6	90	49.3	3.00E-07
BAB80940.1		nagJ	hyaluronidase	<i>Clostridium perfringens</i> str. 13	bbr01g1553	1001	136	288	673	95	239	27	41	24	161	47	3.00E-06
BAB41377.1	SA0157	capN	capsular polysaccharide synthesis enzyme Cap5N	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1582	295	5	161	340	4	188	26	45	34	188	43.9	7.00E-06

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query			Subject			Analysis											
Genebank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Ida	Pos	Gaps	HSP Length	Score	E-value ²
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB79885.1		lipA	probable lipase	<i>Clostridium perfringens</i> str. 13	bbr01g0901	336	83	208	319	57	189	19	46	7	133	43.1	1.00E-05
BAB62495.1		can	probable collagen adhesin	<i>Clostridium perfringens</i> str. 13	bbr01g0103	1368	1105	1229	1502	1052	1228	30	39	56	179	44.7	2.00E-05
BAB80312.1		entD	probable enterotoxin	<i>Clostridium perfringens</i> str. 13	bbr01g1251	635	84	317	267	24	261	23	40	48	260	43.9	2.00E-05
BAB41368.1	SA0148	capE	capsular polysaccharide synthesis enzyme Cap8E	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1582	342	7	173	340	4	187	24	38	23	187	42	3.00E-05
BAB81287.1	CPE1581		probable lipase	<i>Clostridium perfringens</i> str. 13	bbr01g0901	273	53	248	319	71	298	20	42	40	232	41.6	3.00E-05
AAG06865.1	PA3477	rhIR	transcriptional regulator RhIR	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0772	241	181	241	268	208	268	40	55	0	61	37.7	4.00E-04
BAB41367.1	SA0147	capD	capsular polysaccharide synthesis enzyme Cap5D	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1814	607	279	543	340	6	282	22	40	40	291	39.3	4.00E-04
BAB41250.1	SA0032	bleO	bleomycin resistance protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1647	134	14	132	116	5	116	27	44	23	127	35.8	5.00E-04
BAB41369.1	SA0149	capF	capsular polysaccharide synthesis enzyme Cap5F	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0026	369	111	212	735	129	228	28	43	6	104	37.7	6.00E-04
AAG07200.1	PA3813	iscU	probable iron-binding protein IscU	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0865	128	35	91	179	62	120	32	55	2	59	34.3	0.001
BAB43594.1	SA2291	fnb	fibronectin-binding proteins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0180	1038	878	969	337	242	333	29	37	8	96	37.4	0.002
BAB81919.1		cphA	cyanophycin synthetase	<i>Clostridium perfringens</i> str. 13	bbr01g1309	874	479	712	481	102	321	25	41	38	246	37.7	0.002

Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query			Subject			Analysis												
GenBank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos	Gaps	HSP Length	Score	E-value	
Accession Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End							
BAB42897.1	SA1629	spIC	serine protease SpIC	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0079	239	132	207	575	283	370	29	41	20	92	34.7	0.003	
AAG04819.1	PA1430	lasR	transcriptional regulator LasR	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0772	239	160	237	268	188	268	32	46	3	81	33.5	0.006	
BAB42145.1	SA0900	sspB	cysteine protease precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0048	393	264	312	840	321	362	34	53	7	49	34.7	0.006	
BAB42146.1	SA0901	sspA	serine protease; V8 protease; glutamyl endopeptidase	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0079	342	84	252	575	209	371	23	38	36	184	33.5	0.011	
BAB41257.1	SA0039	mecR1	methicillin resistance protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0162	585	44	87	1016	106	149	36	59	0	44	34.3	0.012	
BAB42899.1	SA1631	splA	serine protease SplA	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0079	235	113	203	575	275	370	27	40	23	105	32.7	0.012	
AAG08074.1	PA4687	hitA	ferric iron-binding periplasmic protein HitA	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1587	335	241	278	430	295	332	42	57	0	38	32.7	0.017	
AAA17490.1	L25372		exfoliative toxin A	<i>Staphylococcus aureus</i>	bbr01g0079	280	157	277	575	274	404	25	44	24	138	32.3	0.018	
AAA26625.1	M17347		ETA precursor	<i>Staphylococcus aureus</i>	bbr01g0079	280	157	277	575	274	404	25	44	24	138	32.3	0.018	
AAA26626.1	M17357		epidermolytic toxin A precursor	<i>Staphylococcus aureus</i>	bbr01g0079	280	157	277	575	274	404	25	44	24	138	32.3	0.018	
AAG08290.1	PA4905	vanB	vanillate O-demethylase oxidoreductase	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0918	317	81	208	274	98	226	26	40	9	133	32.3	0.022	
BAB80587.1		nagl	hyaluronidase	<i>Clostridium perfringens</i> str. 13	bbr01g1553	1297	146	272	673	94	221	23	44	7	131	34.3	0.024	
CAA43885.1	X61716		Sphingomyelinase	<i>Staphylococcus aureus</i>	bbr01g1666	331	83	206	434	294	404	20	39	13	124	32.3	0.025	

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query					Subject	Analysis											
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos.	Gaps	HSP Length	Score	E-value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB80516.1		fhuD	probable iron(III) dicitrate ABC transporter	<i>Clostridium perfringens</i> str. 13	bbr01g1528	314	107	182	514	245	324	27	48	10	83	31.6	0.04
AAA53286.1	M28523		enterotoxin	<i>Escherichia coli</i>	bbr01g1078	122	72	109	316	198	241	38	54	6	44	28.9	0.055
BAB80985.1		nagK	hyaluronidase	<i>Clostridium perfringens</i> str. 13	bbr01g0388	1163	321	357	247	145	182	36	63	1	38	33.1	0.056
BAB43626.1	SA2323		conserved hypothetical protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0524	322	166	207	371	177	218	33	59	0	42	30.8	0.057
BAB41326.1	SA0107	spa	Immunoglobulin G binding protein A precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0113	450	266	322	1714	1597	165 1	37	50	6	59	31.2	0.07
BAB79897.1		nagH	hyaluronidase	<i>Clostridium perfringens</i> str. 13	bbr01g0415	1628	1069	121 7	385	16	156	23	37	54	172	33.1	0.07
AAG04821.1	PA1432	lasI	autoinducer synthesis protein LasI	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0143	201	71	138	314	55	130	26	42	8	76	29.6	0.071
AAG08250.1	PA4865	ureA	urease gamma subunit	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0816	100	13	78	350	175	235	27	46	5	66	27.7	0.073
BAB41798.1	SA0566		possible iron permease components	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0544	295	110	167	154	85	145	24	57	3	61	30.4	0.075
AAG08575.1	PA5190		probable nitroreductase	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1858	200	9	191	255	12	182	26	42	20	187	29.6	0.078
AAG08249.1	PA4864	ureD	urease accessory protein	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1357	280	34	116	490	145	227	27	39	22	94	30	0.1
AAA26638.1	L01055		gamma-hemolysin component C	<i>Staphylococcus aureus</i>	bbr01g1360	315	65	112	408	7	56	22	48	2	50	30	0.11
BAB43510.1	SA2208	hlgC	gamma-hemolysin component C	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1360	315	65	112	408	7	56	22	48	2	50	30	0.11

Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query			Description	Species	Accession Number	Analysis											
Query						Query			Subject			E-value	Score	bits	Pos.	Gap	Ident
Accession Number	Gene ID	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
CAA74170.1	Y13859		EscD protein	<i>Escherichia coli</i>	bbr01g0094	406	55	137	544	356	439	26	45	1	84	30.4	0.11
AAA04615.1			Cholera Toxin B subunit	<i>Vibrio cholerae</i>	bbr01g0450	124	1	118	326	159	277	27	42	19	128	27.7	0.12
AAB49627.1	U63134		the 5' end of the open reading frame shows similarity to the rgg protein of <i>Streptococcus gordonii</i> , Swiss-Prot Accession Number P49330	<i>Streptococcus pyogenes</i>	bbr01g0107	252	75	127	628	459	516	32	46	5	58	29.3	0.12
CAA41591.1	X58785		cholera toxin B protein(CTB)	<i>Vibrio cholerae</i>	bbr01g0450	124	1	118	326	159	277	27	42	19	128	27.7	0.12
BAB42895.1	SA1627	splF	serine protease SplF	<i>Staphylococcus aureus subsp. aureus</i> N315	bbr01g0079	239	179	206	575	343	370	46	60	0	28	29.3	0.13
BAB41379.1	SA0159	capP	capsular polysaccharide synthesis enzyme Cap5P	<i>Staphylococcus aureus subsp. aureus</i> N315	bbr01g1212	391	41	93	299	139	189	33	54	2	53	30	0.14
BAA11049.1	D67030		ORF22-a	<i>Clostridium botulinum</i>	bbr01g1164	178	98	153	238	22	77	23	51	0	56	28.5	0.15
CAA74171.1	Y13859		SepL protein	<i>Escherichia coli</i>	bbr01g0115	351	158	194	626	204	249	38	59	11	47	29.6	0.15
BAB41750.1	SA0519	sdrC	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	<i>Staphylococcus aureus subsp. aureus</i> N315	bbr01g0312	953	514	566	515	367	427	38	56	10	62	31.2	0.16
BAB79930.1	CPE0224		ferrichrome ABC transporter	<i>Clostridium perfringens</i> str. 13	bbr01g0494	328	101	132	436	313	340	40	65	4	32	29.6	0.16
CAA23532.1	V00275		enterotoxin subunit A	<i>Escherichia coli</i>	bbr01g1649	254	121	158	334	161	205	33	55	7	45	28.9	0.16
CAA74172.1	Y13859		EspA protein	<i>Escherichia coli</i>	bbr01g0235	192	88	165	879	601	683	26	43	5	83	28.5	0.16

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query					Subject	Analysis											
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Ids	Pos	Gaps	HSP Length	Score	E-value ²
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB42528.1	SA1268	ebhB	probable extra cellular matrix binding proteins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0105	3890	3136	3207	280	31	102	29	45	0	72	33.1	0.17
BAB43376.1	SA2079	fhuD2	hydroxamate siderophore binding lipoprotein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0651	302	138	166	616	172	200	37	65	0	29	29.3	0.17
BAB80970.1	CPE1264		sialidase-like protein	<i>Clostridium perfringens</i> str. 13	bbr01g0154	1588	504	558	422	129	183	27	54	0	55	32	0.17
BAB41611.1	SA0383	set7	exotoxin 7	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1070	231	130	179	205	9	58	30	50	0	50	28.9	0.18
BAB42896.1	SA1628	spID	serine protease SpID	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0079	239	179	206	575	343	370	42	60	0	28	28.9	0.18
BAB81553.1	CPE1847		probable fibronectin-binding protein	<i>Clostridium perfringens</i> str. 13	bbr01g0037	575	426	496	551	443	513	28	50	6	74	30.4	0.18
AAB36016.2	S80809		progenitor toxin L nontoxic-nonhemagglutinin component	<i>Clostridium botulinum</i>	bbr01g1538	1196	482	563	500	110	192	29	44	5	85	31.6	0.19
AAG05004.1	PA1615		probable lipase	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0287	113	26	68	312	96	139	43	54	1	44	26.9	0.19
CAA27568.1	X03929		unnamed protein product; precursor (aa - 30 to 220)	<i>Streptococcus pyogenes</i>	bbr01g1755	250	60	101	542	258	299	33	54	0	42	28.5	0.21
BAB41613.1	SA0385	set9	exotoxin 9	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0682	292	41	74	444	76	111	41	58	2	36	28.9	0.22
CAA43758.1	X61560		type A exotoxin	<i>Streptococcus pyogenes</i>	bbr01g1755	236	52	93	542	258	299	33	54	0	42	28.5	0.22
BAB43097.1	SA1817	sec3	enterotoxin typeC3	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1827	266	2	69	601	28	97	27	52	10	74	28.5	0.23
CAA25801.1	X01645		unnamed protein product;	<i>Staphylococcus aureus</i>	bbr01g0535	319	212	241	707	328	357	36	63	0	30	28.9	0.23

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query			Description	Species	Subject	Analysis											
Genbank Accession Information						B. Breve M-16V Gene ID	Query			Subject			Id%	Pos.	Gaps	HSP Length	Score
Protein ID Number	Query ID Number	Gene Symbol	Length (aa)	Start	End		Length (aa)	Start	End								
			put. alpha-toxin precursor (aa - 26 to 293)														
CAA35972.1	X51661		staphylococcal enterotoxin C3	<i>Staphylococcus aureus</i>	bbr01g1827	266	2	69	601	28	97	27	52	10	74	28.5	0.23
BAB42258.1	SA1007		Alpha-Hemolysin precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0836	319	61	106	891	236	284	32	44	3	49	28.9	0.25
BAB43508.1	SA2206	sbi	IgG-binding protein SBI	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0353	436	20	60	566	372	412	36	53	0	41	29.3	0.25
BAB41751.1	SA0520	sdrD	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0104	1385	836	863	611	491	519	55	65	1	29	31.2	0.26
AAA25805.1	M23348		enzymatically inactive exotoxin A	<i>Pseudomonas aeruginosa</i>	bbr01g0684	129	32	49	1030	138	155	50	77	0	18	26.6	0.28
AAA27528.1	M36855		hemolysin (hlyA)	<i>Vibrio cholerae</i>	bbr01g1368	741	283	366	414	281	365	30	47	3	86	30	0.28
AAG08883.1	PA5498		probable adhesin	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1700	307	229	299	225	105	171	30	46	4	71	28.5	0.28
BAB41346.1	SA0126		probable capsular polysaccharide synthesis protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0322	412	277	322	486	398	439	37	50	8	48	29.3	0.28
BAB41372.1	SA0152	capI	capsular polysaccharide synthesis enzyme Cap5I	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0690	369	238	271	737	314	347	32	61	0	34	28.9	0.28
AAG08657.1	PA5272	cyaA	adenylate cyclase	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1830	950	754	941	438	191	368	25	40	30	198	30.4	0.31
BAB43674.1	SA2369		possible iron transport proteins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1585	446	116	162	316	242	297	26	55	9	56	29.3	0.31

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query			Subject			Analysis											
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Ids	Pos	Gaps	HSP Length	Score	E-value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB42136.1	SA0891		possible ferrichrome ABC transporter	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0460	319	1	39	473	4	44	31	56	2	41	28.5	0.32
BAB42230.1	SA0981	isdF	possible ferrichrome ABC transporter components	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0876	273	164	257	379	66	176	23	40	31	118	28.1	0.35
CAA63386.1	X92727		toxin	<i>Yersinia pestis</i> EV76	bbr01g0685	531	215	271	513	263	323	29	49	4	61	29.3	0.36
BAB42995.1	SA1725		Staphopain, Cysteine Proteinase	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1095	388	96	143	1039	129	177	28	46	1	49	28.5	0.38
BAB41612.1	SA0384	set8	exotoxin 8	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0677	356	284	347	159	59	115	29	51	7	64	28.5	0.39
BAB41978.1	SA0745		extracellular ECM and plasma binding protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1808	173	112	133	750	710	731	54	68	0	22	26.9	0.4
BAB41975.1	SA0742	clfA	fibrinogen-binding protein A, clumping factor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1676	989	2	31	399	93	122	30	66	0	30	30	0.41
BAB41366.1	SA0146	capC	capsular polysaccharide synthesis enzyme Cap8C	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1553	254	6	51	673	390	437	37	45	2	48	27.7	0.42
BAB80964.1		entA	probable enterotoxin	<i>Clostridium perfringens</i> str. 13	bbr01g0968	955	779	816	500	73	108	42	57	2	38	30	0.43
BAB41276.1	SA0058	ccrA	cassette chromosome recombinase A	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0150	449	162	208	320	274	314	34	48	6	47	28.5	0.45
BAB41378.1	SA0158	capO	capsular polysaccharide synthesis enzyme Cap8O	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1747	420	248	299	231	145	204	30	41	8	60	28.5	0.45

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BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query					Subject	Analysis											
General Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos.	Gaps	HSP Length	Score	E- value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB41439.1	SA0217		possible iron-binding protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1459	322	116	162	362	133	176	29	57	3	47	28.1	0.46
BAB41976.1	SA0743		possible staphylocoagulase	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0333	500	392	474	237	120	194	26	39	8	83	28.9	0.47
AAG08198.1	PA4813	lipC	lipase LipC	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1720	309	156	189	639	558	591	35	64	0	34	27.7	0.48
BAB81229.1		nagL	hyaluronidase	<i>Clostridium perfringens</i> str. 13	bbr01g1843	1127	995	1052	609	359	414	31	48	2	58	30	0.48
AAA26628.1	M17348		ETB precursor	<i>Staphylococcus aureus</i>	bbr01g1250	277	166	195	158	98	127	43	53	0	30	27.7	0.5
BAB43735.1	SA2430	aur	zinc metalloproteinase aureolysin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1812	509	318	390	297	98	141	28	36	29	73	28.5	0.5
BAB42898.1	SA1630	splB	serine protease SplB	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0891	240	202	224	511	251	273	52	56	0	23	27.3	0.51
AAA32182.1	K01722		diphtheria toxin (gtg start codon)	<i>Corynebacterium beta</i>	bbr01g0839	560	404	469	208	107	176	31	47	12	74	28.9	0.54
AAA88550.1	M11118		enterotoxin B	<i>Staphylococcus aureus</i>	bbr01g0971	266	128	177	862	372	421	26	48	0	50	27.3	0.54
BAB42253.1	SA1003		possible fibrinogen-binding proteins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0597	165	40	61	396	247	268	50	63	0	22	26.6	0.54
CAA11969.1	AJ224480		C2 toxin (component I)	<i>Clostridium botulinum</i>	bbr01g0332	431	55	147	549	9	93	24	41	16	97	28.1	0.56
AAB59097.1	K01397		exotoxin type A	<i>Pseudomonas aeruginosa</i>	bbr01g0260	638	315	384	222	35	104	27	47	0	70	28.9	0.59
BAB80552.1	CPE0846		alpha-clostripain	<i>Clostridium perfringens</i> str. 13	bbr01g1609	524	339	411	750	26	93	32	49	9	75	28.5	0.6
BAB42906.1	SA1638	lukE	leukotoxin LukE	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0694	311	152	208	318	93	155	31	46	6	63	27.3	0.61

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query					Subject	Analysis											
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Ids	Pos	Gaps	HSP Length	Score	E-value ²
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
AAG08253.1	PA4868	ureC	urease alpha subunit	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0607	566	175	245	1123	944	997	31	43	19	72	28.5	0.63
CAA57277.1	X81586		hlgC-like ORF	<i>Staphylococcus aureus</i>	bbr01g0901	315	214	272	319	87	134	37	49	11	59	27.3	0.63
BAB41622.1	SA0393	set15	exotoxin 15	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0913	227	70	138	1060	210	278	24	49	0	69	26.9	0.64
BAB41310.1	SA0091	plc	1-phosphatidylinositol phosphodiesterase precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1420	328	111	160	345	169	218	28	48	0	50	27.3	0.65
BAB42574.1	SA1312	ebpS	elastin binding protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1317	486	134	221	759	80	168	26	47	7	92	28.1	0.65
CAA85378.1	Z36907		suilysin	<i>Streptococcus suis</i>	bbr01g0607	497	298	415	1123	221	344	22	38	38	140	28.1	0.65
BAB41267.1	SA0049	ant(9)	O-nucleotidyltransferase(9)	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0167	260	120	145	549	487	512	50	65	0	26	26.9	0.66
BAB42002.1	SA0765	ant(9)	O-nucleotidyltransferase(9)	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0167	260	120	145	549	487	512	50	65	0	26	26.9	0.66
BAB42747.1	SA1481	ant(9)	O-nucleotidyltransferase(9)	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0167	260	120	145	549	487	512	50	65	0	26	26.9	0.66
BAB43236.1	SA1952	ant(9)	O-nucleotidyltransferase(9)	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0167	260	120	145	549	487	512	50	65	0	26	26.9	0.66
BAB43690.1	SA2385	ant(9)	O-nucleotidyltransferase(9)	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0167	260	120	145	549	487	512	50	65	0	26	26.9	0.66
CAA63551.1	X92973		bont	<i>Clostridium botulinum</i> A	bbr01g1605	65	19	64	351	32	77	30	45	0	46	24.6	0.7
BAB41614.1	SA0386	set10	exotoxin 10	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0334	234	139	191	431	41	86	28	49	7	53	26.6	0.71
AAC26107.1	AF043556		hemolysin	<i>Streptococcus suis</i>	bbr01g0607	497	298	415	1123	221	344	22	38	38	140	28.1	0.72

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BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query			Subject			Analysis												
Gene/Protein Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos.	Gap%	HSP Length	Score	E-value	
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End							
BAB41752.1	SA0521	sdrE	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1744	1141	191	231	427	140	181	35	59	1	42	29.3	0.73	
BAB42915.1	SA1647	sem	enterotoxin SEM	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1452	239	102	156	502	356	405	30	53	7	56	26.6	0.73	
AAA71894.1	L04539		Shiga-like toxin type-I alpha subunit	<i>Escherichia coli</i>	bbr01g0954	89	35	57	251	167	189	47	65	0	23	24.3	0.74	
AAA98348.1	M19437		Shiga toxin-like subunit B	<i>Shigella dysenteriae</i>	bbr01g0954	89	35	57	251	167	189	47	65	0	23	24.3	0.74	
AAA23283.1	M30307		toxin A	<i>Clostridium difficile</i>	bbr01g0700	2710	1395	1488	703	484	586	24	41	9	103	30.4	0.77	
BAB43593.1	SA2290	fnbB	fibronectin-binding proteins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1653	961	112	232	721	205	339	22	37	16	136	28.9	0.77	
BAB43765.1	SA2460	icaD	intercellular adhesion protein D	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0192	101	24	49	874	583	612	36	56	4	30	24.6	0.77	
AAP31981.1	S76749		toxin	<i>Clostridium botulinum</i>	bbr01g1116	347	275	305	740	48	90	32	46	12	43	27.3	0.8	
BAA08311.1	D45904		lambda toxin	<i>Clostridium perfringens</i>	bbr01g1731	553	357	436	403	151	212	26	40	18	80	28.1	0.8	
AAA91819.1	M24149		cerA	<i>Bacillus cereus</i>	bbr01g0987	283	173	210	230	73	115	29	52	7	44	26.9	0.81	
BAB41370.1	SA0150	capG	capsular polysaccharide synthesis enzyme Cap5G	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1337	374	159	195	275	66	102	35	54	0	37	27.3	0.83	
BAB41979.1	SA0746		staphylococcal nuclease	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1546	228	152	216	1045	306	387	25	42	17	82	26.2	0.89	
BAB41336.1	SA0117	sbnF	siderophore biosynthesis protein SbnF	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1144	592	506	584	780	646	718	33	42	8	80	28.1	0.91	
BAB43036.1	SA1761	sep	enterotoxin P	<i>Staphylococcus aureus</i> subsp.	bbr01g0835	260	149	221	193	81	148	34	52	9	75	26.6	0.91	

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query				Subject	Analysis												
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Ida	Pos	Gaps	HSP Length	Score	E-value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
				<i>aureus</i> N315													
BAB43032.1	SA1758	sak	STAPHYLOKIN ASE PRECURSOR	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0432	163	16	133	368	29	152	22	44	12	127	25.4	0.92
BAB41977.1	SA0744	ssp	extracellular ECM and plasma binding protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0445	340	90	129	396	329	368	27	50	0	40	26.9	0.93
BAB43728.1	SA2423	clfB	Clumping factor B	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0789	877	96	128	289	2	34	36	57	0	33	28.5	0.97
AAC45754.1	U84782		pyolysin	<i>Arcanobacterium pyogenes</i>	bbr01g1088	534	190	244	215	44	97	33	51	3	56	27.7	0.99
BAB80517.1		fhuB	probable ferrichrome ABC transporter	<i>Clostridium perfringens</i> str. 13	bbr01g1359	337	212	263	917	104	168	30	41	13	65	26.9	0.99
BAB41610.1	SA0382	set6	exotoxin 6	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1350	226	147	200	314	139	194	26	48	2	56	26.2	1
AAA53285.1	M28523		enterotoxin	<i>Escherichia coli</i>	bbr01g0665	263	174	193	1006	478	497	50	60	0	20	26.6	1.1
AAB32849.1	S74768		hemagglutinin 70	<i>Clostridium botulinum</i>	bbr01g0638	623	105	166	160	97	158	22	51	0	62	27.7	1.1
AAL32279.1	U84782		hypothetical protein	<i>Arcanobacterium pyogenes</i>	bbr01g1740	352	203	230	297	30	57	39	57	0	28	26.9	1.1
BAB79879.1		colA	collagenase	<i>Clostridium perfringens</i> str. 13	bbr01g1231	1104	107	153	1215	269	315	27	53	0	47	28.9	1.1
BAB79929.1	CPE0223		ferrichrome ABC transporter	<i>Clostridium perfringens</i> str. 13	bbr01g1395	295	43	105	463	391	461	32	44	14	74	26.6	1.1
AAG07199.1	PA3812	iscA	probable iron-binding protein IscA	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1022	107	8	37	800	11	36	40	53	4	30	23.9	1.2
BAB42261.1	SA1009		probable exotoxins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1394	238	55	169	599	453	573	23	41	16	126	26.2	1.2

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BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query					Subject	Analysis												
Gene/Protein Information			Description	Species	Accession ID/ Gene ID	Query			Subject			Id	Pos	Cover	HSP Length	Safety	E-value	
Accession Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End							
AAG08289.1	PA4904	vanA	vanillate O-demethylase oxygenase subunit	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1238	351	35	92	299	125	174	39	53	8	58	26.6	1.3	
CAA37298.1	X53138		unnamed protein product; toxin B (AA 1 - 2366)	<i>Clostridium difficile</i>	bbr01g0227	2366	808	979	392	198	354	23	43	19	174	29.6	1.3	
CAA74173.1	Y13859		EspD protein	<i>Escherichia coli</i>	bbr01g0789	380	315	349	289	161	195	37	54	0	35	26.6	1.3	
AAA27569.1	M10069		prethermostable direct hemolysin	<i>Vibrio parahaemolyticus</i>	bbr01g1058	189	20	70	261	29	69	31	49	10	51	25.4	1.4	
AAA71893.1	L04539		Shiga-like toxin type-I alpha subunit	<i>Escherichia coli</i>	bbr01g0530	315	228	287	341	69	128	28	41	0	60	26.2	1.4	
AAG08252.1	PA4867	ureB	urease beta subunit	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0957	101	7	38	354	66	101	41	55	4	36	23.5	1.4	
BAB42527.1	SA1267	ebhA	probable extracellular matrix binding proteins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0804	6713	4194	4226	475	225	257	42	63	0	33	31.2	1.4	
BAB42913.1	SA1645	yent1	enterotoxin Yent1	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0910	133	68	125	767	226	279	34	51	4	58	24.6	1.4	
AAA91820.1	M24149		cerB	<i>Bacillus cereus</i>	bbr01g1087	333	147	160	279	208	221	64	78	0	14	26.2	1.5	
AAA98347.1	M19437		Shiga toxin-like subunit A	<i>Shigella dysenteriae</i>	bbr01g0530	315	228	287	341	69	128	28	41	0	60	26.2	1.5	
AAA99192.1	L43545		alpha-toxin(phospholipase C)	<i>Clostridium perfringens</i>	bbr01g1154	398	11	99	320	146	228	27	39	14	93	26.6	1.5	
AAA99193.1	L43546		alpha-toxin(phospholipase C)	<i>Clostridium perfringens</i>	bbr01g1154	398	11	99	320	146	228	27	39	14	93	26.6	1.5	
BAB41615.1	SA0387	set11	exotoxin 11	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1053	231	5	41	438	312	348	32	59	0	37	25.8	1.5	
BAB41799.1	SA0567		possible iron permease	<i>Staphylococcus aureus</i> subsp.	bbr01g0226	316	220	259	191	26	65	30	55	0	40	26.2	1.5	

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																		
Query					Subject	Analysis												
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos	Gaps	HSP Length	Score	E-value ²	
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End							
			components	<i>aureus</i> N315														
BAB43096.1	SA1816	sel	extracellular enterotoxin L	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1533	240	65	139	359	228	302	17	38	0	75	25.8	1.5	
BAB79742.1		plc	phospholipase C	<i>Clostridium perfringens</i> str. 13	bbr01g1154	398	11	99	320	146	228	27	39	14	93	26.6	1.5	
AAA96668.1	L77573		cytotoxic enterotoxin	<i>Aeromonas hydrophila</i>	bbr01g1371	368	202	225	488	81	104	41	58	0	24	26.6	1.6	
BAB41335.1	SA0116	sbnE	siderophore biosynthesis protein SbnE	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0202	578	350	396	282	55	101	27	53	0	47	27.3	1.6	
BAB42263.1	SA1011		probable exotoxins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1662	241	35	59	370	190	214	40	56	0	25	25.4	1.6	
AAA23234.1	M31795		type A enterotoxin	<i>Clostridium perfringens</i>	bbr01g0197	66	21	48	340	158	185	46	53	0	28	23.1	1.7	
AAA24686.1	M29255		heat-stable toxin	<i>Escherichia coli</i>	bbr01g1916	72	4	47	967	404	447	25	47	0	44	23.1	1.7	
BAB42144.1	SA0899	sspC	cysteine protease	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1812	109	25	45	297	109	129	38	57	0	21	23.5	1.7	
BAB41251.1	SA0033	aadD	kanamycin nucleotidyltransferase	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1874	256	11	33	690	584	606	34	78	0	23	25.4	1.8	
AAA23270.1	M36704		perfringolysin O	<i>Clostridium perfringens</i>	bbr01g1191	499	295	491	530	210	383	24	36	35	203	26.6	1.9	
BAB79869.1		pfoA	perfringolysin O	<i>Clostridium perfringens</i> str. 13	bbr01g1191	500	296	492	530	210	383	23	35	35	203	26.6	1.9	
CAA28033.1	X04436		tetanus toxin precursor(AA 1-1315)	<i>Clostridium tetani</i>	bbr01g1597	1315	102	179	232	86	165	31	43	2	80	28.1	2	
CAA74174.1	Y13859		EspB protein	<i>Escherichia coli</i>	bbr01g1268	314	51	144	397	12	107	25	45	18	104	25.8	2	
AAA20995.1	U10527		dermonecrotic toxin	<i>Bordetella pertussis</i>	bbr01g0026	1451	841	880	735	643	682	30	57	0	40	28.1	2.1	

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BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query			Description	Species	B. Breve M-16V Gene ID	Analysis											
Database Accession Information						Query			Subject			Id%	Pos	Clust	HSP Length	Score	E-value
Accession Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB42262.1	SA1010		probable exotoxins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0101	241	120	153	647	520	550	44	50	3	34	25.4	2.1
BAB42979.1	SA1709		possible ferritin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1154	166	127	164	320	59	98	32	47	2	40	24.3	2.1
AAA26637.1	L01055		gamma-hemolysin component A	<i>Staphylococcus aureus</i>	bbr01g1009	309	247	269	175	11	33	43	56	0	23	25.8	2.2
AAA72120.1	M98037		enterotoxin	<i>Clostridium perfringens</i>	bbr01g1076	319	272	288	987	605	621	64	76	0	17	25.8	2.2
AAA99194.1	L43547		alpha-toxin(phospholipase C)	<i>Clostridium perfringens</i>	bbr01g1154	398	11	99	320	146	228	27	39	14	93	26.2	2.2
BAA07714.1	D42143		gamma-hemolysin	<i>Staphylococcus aureus</i>	bbr01g1009	309	247	269	175	11	33	43	56	0	23	25.8	2.2
BAB43509.1	SA2207	hlgA	gamma-hemolysin chain II precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1009	309	247	269	175	11	33	43	56	0	23	25.8	2.2
BAB43769.1	SA2463	lip	triacylglycerol lipase precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0257	681	306	371	661	546	611	25	43	0	66	26.9	2.2
CAA04327.1	AJ000766		enterotoxin	<i>Clostridium perfringens</i>	bbr01g1076	319	272	288	987	605	621	64	76	0	17	25.8	2.2
CAA75931.1	Y16009		enterotoxin	<i>Clostridium perfringens</i>	bbr01g1076	319	272	288	987	605	621	64	76	0	17	25.8	2.2
AAG07111.1	PA3724	lasB	elastase LasB	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1608	498	116	133	266	149	166	66	72	0	18	26.6	2.3
BAA04104.1	D16824		hemolysin	<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i>	bbr01g1054	574	15	53	561	6	48	34	53	4	43	26.6	2.4
BAB42916.1	SA1648	seo	enterotoxin SeO	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0246	260	160	208	104	21	72	26	53	3	52	25	2.4
AAC08437.1	AF053400		truncated toxin A	<i>Clostridium difficile</i>	bbr01g1927	698	585	635	339	134	184	25	52	0	51	26.9	2.5

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BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query			Subject	Analysis													
Genebank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Ids	Pos	Gaps	HSP Length	Score	E-value ²
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
AAG05579.1	PA2191	exoY	adenylate cyclase ExoY	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1305	378	13	65	224	96	153	28	49	7	59	25.8	2.5
BAB41374.1	SA0154	capK	capsular polysaccharide synthesis enzyme Cap5K	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0784	401	351	375	460	60	86	44	74	2	27	25.8	2.5
BAB42254.1	SA1004		possible fibrinogen-binding proteins	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1093	116	39	70	422	216	247	37	62	0	32	23.1	2.5
AAA64889.1	M85198		NAG-ST	<i>Vibrio cholerae</i>	bbr01g0962	78	6	48	443	379	424	26	56	3	46	22.7	2.6
BAA04105.1	D16825		hemolysin	<i>Streptococcus canis</i>	bbr01g1054	574	15	53	561	6	48	34	53	4	43	26.6	2.6
BAB41616.1	SA0388	set12	exotoxin 12	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0831	232	31	82	278	188	243	28	42	4	56	25	2.6
AAA99195.1	L43548		alpha-toxin(phospholipase C)	<i>Clostridium perfringens</i>	bbr01g1154	398	11	99	320	146	228	27	39	14	93	25.8	2.8
BAB43122.1	SAS065	hld	delta-hemolysin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0080	44	11	44	822	372	405	29	47	0	34	22.3	2.8
BAB41258.1	SA0040	mecl	methicillin resistance regulatory protein	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0958	123	94	120	148	81	107	37	59	0	27	23.1	3.1
BAB41373.1	SA0153	capJ	capsular polysaccharide synthesis enzyme Cap5J	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1920	388	299	327	309	131	163	30	63	4	33	25.4	3.3
BAB42229.1	SA0980	isdE	possible ferrichrome ABC transporter components	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0979	292	132	162	252	54	85	37	65	1	32	25	3.3
AAA23284.1	L13198		beta-toxin	<i>Clostridium perfringens</i>	bbr01g1080	336	28	72	423	238	282	20	48	0	45	25	3.4
AAA26975.1	M18638		streptolysin O precursor(put.;	Plasmid pMK157	bbr01g1467	571	263	350	671	539	622	23	46	4	88	26.2	3.4

Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query			Subject			Analysis											
Database Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos.	Gaps	HSPs/Length	Score	E-value
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
			start may be at 190); putative														
AAL32276.1	U84782		hypothetical protein	<i>Arcanobacterium pyogenes</i>	bbr01g0095	120	16	53	350	129	166	34	44	0	38	23.1	3.4
BAB80443.1	CPE0737		probable fibronectin-binding protein	<i>Clostridium perfringens</i> str. 13	bbr01g0925	220	120	138	533	349	367	52	68	0	19	24.3	3.4
CAA80815.1	Z23277		toxin B	<i>Clostridium difficile</i>	bbr01g0900	2367	1630	1724	282	182	276	21	41	0	95	28.1	3.4
AAA26915.1	M17717		pneumolysin	<i>Streptococcus pneumoniae</i>	bbr01g0548	471	322	383	290	34	95	30	43	0	62	25.8	3.5
CAA63550.1	X92973		ntnh	<i>Clostridium botulinum</i> A	bbr01g0316	1193	917	950	902	606	637	41	55	2	34	27.3	3.5
BAA11050.1	D67030		type A progenitor toxin nontoxic-nonHA	<i>Clostridium botulinum</i>	bbr01g0316	1193	917	950	902	606	637	41	55	2	34	26.9	3.6
CAA37321.1	X53180		botulinum type E toxin	<i>Clostridium butyricum</i>	bbr01g0573	252	46	100	1127	227	281	25	41	0	55	24.6	3.7
AAA26617.1	M21319		enterotoxin type E precursor	<i>Staphylococcus aureus</i>	bbr01g1033	257	9	41	561	16	48	33	63	0	33	24.6	3.9
AAG06864.1	PA3476	rhII	autoinducer synthesis protein RhII	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g1738	201	175	194	411	167	186	35	65	0	20	23.9	3.9
BAB42912.1	SA1644	yent2	enterotoxin YENT2	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0115	136	21	39	626	406	424	42	73	0	19	23.1	4
BAB41617.1	SA0389	set13	exotoxin 13	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0665	232	36	60	1006	692	716	40	56	0	25	24.3	4.2
BAB42914.1	SA1646	sei	extracellular enterotoxin type I precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0785	242	148	173	747	186	212	42	67	3	28	24.3	4.2
AAA26681.1	M18970		staphylococcal enterotoxin A precursor	<i>Staphylococcus aureus</i>	bbr01g1033	257	9	41	561	16	48	33	63	0	33	24.3	4.5
AAA23236.1	M95206		epsilon-toxin	<i>Clostridium perfringens</i>	bbr01g0153	328	231	318	763	372	466	27	40	9	96	24.6	4.6

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by <i>Bifidobacterium breve</i> (B. breve) M-16V ¹																	
Query					Subject	Analysis											
Genbank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Ida.	Pos.	Gaps	HSP Length	Score	E-value ²
Protein ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
BAB43093.1	SA1813		possible leukocidin lukM	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0927	351	4	31	94	23	50	42	57	0	28	24.6	4.6
BAB80158.1		entC	probable enterotoxin	<i>Clostridium perfringens</i> str. 13	bbr01g1898	625	165	223	375	309	358	28	42	9	59	25.8	4.8
CAA29260.1	X05815		unnamed protein product; enterotoxin C1 precursor (AA - 27 to 239)	<i>Staphylococcus aureus</i>	bbr01g1761	266	130	200	448	171	254	28	42	19	87	24.3	4.9
BAB42417.1	SA1160	nuc	thermonucleas e	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0403	177	83	120	478	344	379	34	50	2	38	23.5	5
AAA23235.1	M80837		epsilon-toxin	<i>Clostridium perfringens</i>	bbr01g0153	328	231	318	763	372	466	27	40	9	96	24.6	5.1
AAG06605.1	PA3217		probable adenylate cyclase	<i>Pseudomonas aeruginosa</i> PAO1	bbr01g0383	463	419	442	354	56	79	41	58	0	24	25	5.2
BAB42694.1	SA1430		probable enterotoxin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1718	157	51	77	3160	1498	1524	33	55	0	27	23.1	5.3
BAB43879.1	SAP010	blaZ	beta-lactamase	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0053	281	212	241	349	218	246	33	60	1	30	24.3	5.5
BAB41375.1	SA0155	capL	capsular polysaccharide synthesis enzyme Cap5L	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0078	401	283	314	437	247	278	37	62	0	32	24.6	6.2
BAB41533.1	SA0309	geh	glycerol ester hydrolase	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0700	691	433	478	703	580	629	36	50	4	50	25.4	6.2
BAB41444.1	SA0222	coa	staphylocoagulase precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0923	658	40	75	209	70	105	33	52	0	36	25.4	6.5
BAB43092.1	SA1812		possible hemolysin	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0509	338	25	133	691	285	385	23	39	8	109	24.3	6.5
BAB42905.1	SA1637	lukD	leukotoxin, LukD	<i>Staphylococcus aureus</i> subsp.	bbr01g0937	327	37	80	401	200	242	31	52	1	44	24.3	6.7

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Appendix 8A

BLASTP Analysis of Potential Toxic and Pathogenic Genes Expressed by *Bifidobacterium breve* (B. breve) M-16V¹

Query			Subject		Analysis												
Genebank Accession Information			Description	Species	B. Breve M-16V Gene ID	Query			Subject			Id%	Pos.	Gaps	MSD Length	Score	E-value ²
Accession ID Number	Gene ID Number	Gene Symbol				Length (aa)	Start	End	Length (aa)	Start	End						
				<i>aureus</i> N315													
BAB62455.1		cpb2	beta2-toxin	<i>Clostridium perfringens</i> str. 13	bbr01g0738	265	28	48	825	325	345	38	85	0	21	23.5	7.3
BAB42911.1	SA1643	sen	enterotoxin SeN	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g0749	258	181	203	791	551	573	39	56	0	23	23.5	8.2
BAB43294.1	SA2003	hysA	hyaluronate lyase precursor	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315	bbr01g1076	809	617	667	987	177	227	25	49	0	51	25.4	8.3
CAA55074.1	X78230		nontoxic-nonhemagglutinin	<i>Clostridium botulinum</i>	bbr01g1824	1197	994	1062	300	109	160	24	43	17	69	25.8	8.8
AAB32848.1	S74768		hemagglutinin	<i>Clostridium botulinum</i>	bbr01g0074	146	103	131	1068	826	854	31	55	0	29	21.9	9.5

¹Amino acid sequences of predicted proteins were compared to the amino acid sequences of known bacterial toxins using BLASTP and Genebank.
²E-value of 0 to 1E-100 equals high homology; E-value of 1E-100 to 1E-50 equals medium homology; E-value of 1E-50 to 1E-30 equals low homology; E-values of >1E-30 equals no homology.

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BLASTP Analysis of Highly Homologous and Potentially Toxic and Pathogenic Gene Products in <i>Bifidobacterium breve</i> (<i>B. breve</i>),											
Homology between <i>B. breve</i> M-16V gene products and those expressed by toxic or pathogenic bacteria (From Appendix 9A)				Homology to other strains for <i>Bifidobacterium</i> (E-value ²)							
Homologous gene products expressed by toxic/pathogenic bacteria ¹	Definition	<i>B. breve</i> M-16V gene	E-Value ²	<i>B. infantis</i>	<i>B. longum</i>	<i>B. bifidum</i>	<i>B. catenulatum</i>	<i>B. adolescentis</i>	<i>B. bifidum</i>	<i>B. breve</i>	<i>B. pseudocatenulatum</i>
AAG07658.1, BAB41731.1, BAB82119.1	RNA polymerase beta chain	bbr01g0428	0	0	0	0	0	0	NF ³	0	0
BAB41321.1.BA B80084.1	myosin-cross reactive protein	bbr01g1344	0	0	0	0	0	0	NF	0	0
AAL32278.1	signal recognition particle protein subunit	bbr01g0330	1E-172	0	0	0	0	0	0	0	0
AAL32277.1	signal recognition particle receptor	bbr01g0203	1E-110	<1E-122	<1E-122	<1E-122	<1E-122	<1E-122	<1E-122	0	6.69E-122
AAL32275.1	chromosome segregation protein	bbr01g1231	6E-79	0	0	2.50E-102	4.33E-03	5.29E-02	NF	0	3.32E-02
BAB80431.1, BAB80259.1	exo-alpha-sialidase	bbr01g0153	6E-63, 6.00E-56	0	> 0.1	4.22E-17	>0.1	>0.1	NF	0	>0.1
BAB81588.1	probable collagenase	bbr01g1703	3E-58	0	0	0	0	0	NF	0	0
BAB80143.1, BAB41890.1, BAB79736.1	probable hemolysin	bbr01g0019	1E-53, 1.00E-44, 1E-48	0	0	0	5.24E-18	0	NF	0	0
BAB41593.1, AAG03529.1	alkyl hydroperoxide reductase subunit C	bbr01g0024	1E-53, 7.00E-51	<1E-100	<1E-100	<1E-100	1.00E-100	2.03E-02	NF	<1E-100	<1E-100
AAG07656.1, BAB82116.1, BAB41734.1	30S ribosomal protein S12	bbr01g0533	2E-49, 2.00E-49, 2.00E-48	<1.0E-50	<1.0E-50	<1.0E-50	<1.0E-50	NF	NF	<1.0eE50	<1.0E-50
BAB81524.1	probable	bbr01g0647	4E-43	<1.0E-84	<1.0E-84	<1.0E-84	<1.0E-84	<1.0E-84	<1.0E-84	<1.0E-84	<1.0E-84

000225

BLASTP Analysis of Highly Homologous and Potentially Toxic and Pathogenic Gene Products in <i>Bifidobacterium breve</i> (<i>B. breve</i>),											
Homology between <i>B. breve</i> M-10V gene products and those encoded by toxic or pathogenic bacteria (From Appendix 9A)				Homology to other strains for <i>Bifidobacterium</i> (E-value) ²							
Homologous gene products encoded by toxic/pathogenic bacteria	Description	<i>B. breve</i> M-10V gene	E-Value	<i>B. infantis</i>	<i>B. longum</i>	<i>B. bifidum</i>	<i>B. caelestium</i>	<i>B. adolescentis</i>	<i>B. bifidum</i>	<i>B. breve</i>	<i>B. probatum</i>
	hemolysin						84			84	
BAB41500.1	diarrheal toxin-like protein	bbr01g0681	2E-41	<2.7E-120	<2.7E-120	<2.7E-120	6.06E-111	2.48E-112	<2.7E-120	<2.7E-120	9.36E-110

¹Homologous gene products were obtained from the BLASTP Analysis in Appendix 9A and Amino acid sequences of predicted proteins were compared to the amino acid sequences of known bacterial toxins using BLASTP and Genebank.
²E-value of 0 to 1E-100 equals high homology; E-value of 1E-100 to 1E-50 equals medium homology; E-value of 1E-50 to 1E-30 equals low homology; E-values of >1E-30 equals little to no homology.
³NF denotes "not found."

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Appendix 9

BLASTP results of g1373, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Sources	% Identity	Score	E-value ²
291457490	ZP_06596880.1	conserved hypothetical protein	Bifidobacterium breve DSM 20213	100	544	0
384196688	YP_005582432.1	unnamed protein product	Bifidobacterium breve ACS-071-V-Sch8b	100	543	0
23464674	NP_695277.1	hypothetical protein BL0046	Bifidobacterium longum NCC2705	90.04	493	6E-175
227546775	ZP_03976824.1	nitroreductase	Bifidobacterium longum subsp. infantis ATCC 55813	90.04	493	8E-175
46190638	ZP_00121302.2	Nitroreductase	Bifidobacterium longum DJO10A	89.66	492	2E-174
384201097	YP_005586844.1	unnamed protein product	Bifidobacterium longum subsp. longum KACC 91563	89.66	492	2E-174
294786742	ZP_06751996.1	nitroreductase family protein	Parascardovia denticolens F0305	47.62	243	1E-76
15894035	NP_347384.1	unnamed protein product	Clostridium acetobutylicum ATCC 824	37.4	169	7E-48
309775082	ZP_07670095.1	nitroreductase family protein	Erysipelotrichaceae bacterium 3_1_53	32.42	140	7E-37
300727386	ZP_07060800.1	nitroreductase family protein	Prevotella bryantii B14	33.86	136	2E-35
293375510	ZP_06621787.1	conserved hypothetical protein	Turicibacter sanguinis PC909	32.08	136	2E-35
325840230	ZP_08166997.1	hypothetical protein HMPREF9402_1542	Turicibacter sp. HGF1	31.67	134	1E-34
374308483	YP_005054914.1	unnamed protein product	Filifactor alocis ATCC 35896	33.46	134	2E-34
317473480	ZP_07932772.1	hypothetical protein HMPREF1011_03122	Anaerostipes sp. 3_2_56FAA	32.67	132	5E-34
306819723	ZP_07453383.1	nitroreductase	Eubacterium yurii subsp. margaretae ATCC 43715	31.4	132	8E-34
167747743	ZP_02419870.1	hypothetical protein ANACAC_02464	Anaerostipes caccae DSM 14662	33.07	131	2E-33
283768792	ZP_06341703.1	conserved hypothetical protein	Bulleidia extracta W1219	31.37	131	2E-33
319900575	YP_004160303.1	nitroreductase	Bacteroides helcogenes P 36-108	32.52	129	6E-33

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BLASTP results of g1373, a potential nitroreductase expressed by *Bifidobacterium breve* M-16V (list of top 100)¹

GenInfo (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value ²
358065480	ZP_09152020.1	hypothetical protein HMPREF9473_04083	Clostridium hathewayi WAL-18680	32.51	126	6E-32
255280695	ZP_05345250.1	nitroreductase family protein	Bryantella formatexigens DSM 14469	32.66	125	2E-31
257064989	YP_003144661.1	nitroreductase	Slackia heliotrinireducens DSM 20476	34.48	126	2E-31
300727300	ZP_07060714.1	nitroreductase family protein	Prevotella bryantii B14	32.67	124	4E-31
346314935	ZP_08856452.1	hypothetical protein HMPREF9022_02109	Erysipelotrichaceae bacterium 2_2_44A	30.43	124	5E-31
315926066	ZP_07922266.1	nitroreductase	Pseudoramibacter alactolyticus ATCC 23263	30.47	124	6E-31
225574222	ZP_03782832.1	hypothetical protein RUMHYD_02286	Blautia hydrogenotrophica DSM 10507	31.2	123	9E-31
313900802	ZP_07834292.1	conserved hypothetical protein	Clostridium sp. HGF2	30.83	124	1E-30
320528159	ZP_08029324.1	nitroreductase family protein	Solobacterium moorei F0204	31.33	122	3E-30
281421596	ZP_06252595.1	nitroreductase family protein	Prevotella copri DSM 18205	32.8	121	8E-30
225016625	ZP_03705817.1	hypothetical protein CLOSTMETH_00532	Clostridium methylpentosum DSM 5476	30.77	120	2E-29
374624294	ZP_09696711.1	hypothetical protein HMPREF0978_00031	Coprobacillus sp. 8_2_54BFAA	31.45	119	2E-29
167756101	ZP_02428228.1	hypothetical protein CLORAM_01621	Clostridium ramosum DSM 1402	31.45	119	2E-29
237734085	ZP_04564566.1	nitroreductase	Mollicutes bacterium D7	31.45	119	3E-29
256827090	YP_003151049.1	nitroreductase	Cryptobacterium curtum DSM 15641	34.85	119	3E-29
160879506	YP_001558474.1	hypothetical protein Cphy_1360	Clostridium phytofermentans ISDg	30	118	1E-28
163815754	ZP_02207126.1	hypothetical protein COPEUT_01935	Coprococcus eutactus ATCC 27759	34.18	116	3E-28
257065108	YP_003144780.1	nitroreductase	Slackia heliotrinireducens DSM 20476	31.8	115	2E-27
295099618	CBK88707.1	hypothetical protein	Eubacterium cylindroides T2-87	29.71	111	2E-26

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Appendix 9

BLASTP results of g1373, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Sources	% Identity	Score	E-value ²
219668168	YP_002458603.1	nitroreductase	Desulfitobacterium hafniense DCB-2	31.6	112	2E-26
221194690	ZP_03567747.1	nitroreductase family protein	Atopobium rimae ATCC 49626	30.71	112	3E-26
167750792	ZP_02422919.1	hypothetical protein EUBSIR_01774	Eubacterium siraeum DSM 15702	33.06	110	8E-26
291530711	CBK96296.1	Nitroreductase family	Eubacterium siraeum 70/3	33.06	110	8E-26
229827999	ZP_04454068.1	hypothetical protein GCWU000342_00048	Shuttleworthia satelles DSM 14600	32.37	109	1E-25
374316643	YP_005063071.1	unnamed protein product	Sphaerochaeta pleomorpha str. Grapes	30.53	109	2E-25
291557779	CBL34896.1	Nitroreductase family	Eubacterium siraeum V10Sc8a	32.65	108	2E-25
89893795	YP_517282.1	hypothetical protein DSY1049	Desulfitobacterium hafniense Y51	30.8	107	8E-25
335045198	ZP_08538221.1	hypothetical protein HMPREF9124_2268	Oribacterium sp. oral taxon 108 str. F0425	29.27	107	1E-24
260439137	ZP_05792953.1	nitroreductase family protein	Butyrivibrio crossotus DSM 2876	28.28	106	2E-24
302336278	YP_003801485.1	nitroreductase	Olsenella uli DSM 7084	30.29	105	4E-24
169825211	YP_001692822.1	putative nitroreductase	Finegoldia magna ATCC 29328	28.51	104	7E-24
302380466	ZP_07268934.1	conserved hypothetical protein	Finegoldia magna ACS-171-V-Co13	31.25	104	9E-24
169640151	ACA61155.1	nitroreductase family protein, partial	uncultured microorganism	29.58	104	1E-23
330836540	YP_004411181.1	unnamed protein product	Spirochaeta coccoides DSM 17374	31.45	104	1E-23
302392258	YP_003828078.1	unnamed protein product	Acetohalobium arabaticum DSM 5501	28.86	104	1E-23
341592162	EGS35048.1	hypothetical protein HMPREF9489_1771	Finegoldia magna SY403409CC001050417	28.74	103	1E-23
303234622	ZP_07321256.1	conserved hypothetical protein	Finegoldia magna BVS033A4	28.63	103	2E-23
310287579	YP_003938837.1	nitroreductase	Bifidobacterium bifidum S17	29.8	103	3E-23
387133375	YP_006299347.1	hypothetical protein PIN17_A1468	Prevotella intermedia 17	27.49	102	3E-23

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BLASTP results of g1373, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenBank (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value
257784997	YP_003180214.1	nitroreductase	Atopobium parvulum DSM 20469	28.57	103	4E-23
363898321	ZP_09324855.1	hypothetical protein HMPREF9624_01417	Oribacterium sp. ACB7	28.46	101	2E-22
355574191	ZP_09044034.1	hypothetical protein HMPREF1008_00011	Olsenella sp. oral taxon 809 str. F0356	30.28	102	2E-22
374581992	ZP_09655086.1	nitroreductase family protein	Desulfosporosinus youngiae DSM 17734	36.08	99.8	3E-22
373494580	ZP_09585183.1	hypothetical protein HMPREF0380_00821	Eubacterium infirmum F0142	27.42	100	3E-22
343521061	ZP_08758029.1	nitroreductase domain protein	Parvimonas sp. oral taxon 393 str. F0440	28.4	99.8	6E-22
384196865	YP_005582609.1	unnamed protein product	Bifidobacterium breve ACS-071-V-Sch8b	30.83	98.6	1E-21
363899923	ZP_09326429.1	hypothetical protein HMPREF9625_01089	Oribacterium sp. ACB1	27.49	99.4	1E-21
297587905	ZP_06946549.1	probable nitroreductase	Finegoldia magna ATCC 53516	28.11	98.2	2E-21
339479347	ABE95815.1	Conserved hypothetical protein	Bifidobacterium breve UCC2003	30.83	98.2	2E-21
319938164	ZP_08012562.1	nitroreductase	Coprobacillus sp. 29_1	27.89	97.8	3E-21
342215982	ZP_08708629.1	nitroreductase domain protein	Peptoniphilus sp. oral taxon 375 str. F0436	28.75	95.5	1E-20
291456931	ZP_06596321.1	nitroreductase family protein	Bifidobacterium breve DSM 20213 = JCM 1192	31.3	95.5	1E-20
365133438	ZP_09342773.1	hypothetical protein HMPREF1032_00569	Subdoligranulum sp. 4_3_54A2FAA	26.75	94.7	2E-20
160939059	ZP_02086410.1	hypothetical protein CLOBOL_03953	Clostridium bolteae ATCC BAA-613	27.52	91.3	5E-19
239627032	ZP_04670063.1	sensor histidine kinase/response regulator	Clostridiales bacterium 1_7_47_FAA	28.91	90.5	9E-19
357053084	ZP_09114187.1	hypothetical protein HMPREF9467_01159	Clostridium clostridioforme 2_1_49FAA	27.06	90.1	1E-18

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BLASTP results of g1373, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100)¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Sources	% Identity	Score	E-value
354558568	ZP_08977823.1	nitroreductase	Desulfitobacterium metallireducens DSM 15288	26.17	89	6E-18
227873788	ZP_03992015.1	nitroreductase family protein	Oribacterium sinus F0268	26.4	87.4	1E-17
366164402	ZP_09464157.1	nitroreductase family protein	Acetivibrio cellulolyticus CD2	24.8	87.4	1E-17
334341148	YP_004546128.1	unnamed protein product	Desulfotomaculum ruminis DSM 2154	25.4	87.4	2E-17
225182034	ZP_03735465.1	nitroreductase	Dethiobacter alkaliphilus AHT 1	28.57	86.7	3E-17
294790995	ZP_06756153.1	putative Nitroreductase family protein	Scardovia inopinata F0304	27.98	84	2E-16
338810719	ZP_08622959.1	nitroreductase	Acetonema longum DSM 6540	28	83.2	5E-16
269216833	ZP_06160687.1	Nitroreductase family protein	Slackia exigua ATCC 700122	29.32	81.6	8E-16
261415319	YP_003249002.1	nitroreductase	Fibrobacter succinogenes subsp. succinogenes S85	27.46	81.6	1E-15
374381426	ZP_09639015.1	nitroreductase	Desulfitobacterium dichloroeliminans LMG P-21439	26.22	82	1E-15
323340072	ZP_08080338.1	hypothetical protein HMPREF0542_10769	Lactobacillus ruminis ATCC 25644	27.57	81.3	1E-15
340359493	ZP_08681978.1	hypothetical protein HMPREF9062_1103	Actinomyces sp. oral taxon 448 str. F0400	27.38	81.3	1E-15
227872464	ZP_03990805.1	nitroreductase family protein	Oribacterium sinus F0268	28.86	80.9	2E-15
363899320	ZP_09325830.1	hypothetical protein HMPREF9625_00490	Oribacterium sp. ACB1	26.34	80.9	2E-15
363897349	ZP_09323888.1	hypothetical protein HMPREF9624_00450	Oribacterium sp. ACB7	25.93	79.3	6E-15
218282774	ZP_03488956.1	hypothetical protein EUBIFOR_01542	Eubacterium bifforme DSM 3989	27.46	79.3	6E-15
229826652	ZP_04452721.1	hypothetical protein GCWU000182_02028	Abiotrophia defectiva ATCC 49176	26.56	79.3	7E-15
335045920	ZP_08538943.1	hypothetical protein HMPREF9124_1362	Oribacterium sp. oral taxon 108 str. F0425	26.64	79	8E-15

Appendix 9

BLASTP results of g1373, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100)¹						
Genbank (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value²
312143325	YP_003994771.1	nitroreductase family protein	Halanaerobium hydrogeniformans	24.43	74.7	3E-13
254519621	ZP_05131677.1	nitroreductase	Clostridium sp. 7_2_43FAA	24.6	71.6	6E-12
188586178	YP_001917723.1	nitroreductase	Natranaerobius thermophilus JW/NM-WN-LF	24.42	71.6	7E-12
297204981	ZP_06922377.1	possible nitroreductase	Lactobacillus jensenii JV-V16	23.89	70.1	1E-11
373107128	ZP_09521428.1	hypothetical protein HMPREF9623_01092	Lachnospiraceae bacterium ACC2	27.13	69.3	2E-11
385800178	YP_005836582.1	unnamed protein product	Halanaerobium praevalens DSM 2228	25.1	70.1	2E-11
256852140	ZP_05557527.1	nitroreductase	Lactobacillus jensenii 27-2-CHN	23.98	69.3	3E-11
219668618	YP_002459053.1	nitroreductase	Desulfitobacterium hafniense DCB-2	25.58	69.7	4E-11

¹The open reading frame of g1373 was determined as described {Kosuge et al., 2006, DNA Res, 13, 245-54} and BLASTed against the NCBI data base (nr, 20120604).
²E-value of 0 to 1E-100 equals high homology; E-value of 1E-100 to 1E-50 equals medium homology; E-value of 1E-50 to 1E-30 equals low homology; E-values of >1E-30 equals no homology.

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BLASTP results of g1083, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Sources	% Identity	Score	E-value ²
339479347	ABE95815.1	Conserved hypothetical protein	Bifidobacterium breve UCC2003	100.0	474	3.0E-168
291456931	ZP_06596321.1	nitroreductase family protein	Bifidobacterium breve DSM 20213	100.0	462	1.0E-163
384196865	YP_005582609.1	unnamed protein product	Bifidobacterium breve ACS-071-V-Sch8b	99.6	473	1.0E-167
310287579	YP_003938837.1	nitroreductase	Bifidobacterium bifidum S17	63.9	303	8.0E-101
225016625	ZP_03705817.1	hypothetical protein CLOSTMETH_00532	Clostridium methylpentosum DSM 5476	43.7	182	1.0E-53
358065480	ZP_09152020.1	hypothetical protein HMPREF9473_04083	Clostridium hathewayi WAL-18680	42.9	175	4.0E-51
225574222	ZP_03782832.1	hypothetical protein RUMHYD_02286	Blautia hydrogenotrophica DSM 10507	39.0	170	6.0E-49
319900575	YP_004160303.1	nitroreductase	Bacteroides helcogenes P 36-108	38.5	162	5.0E-46
291530711	CBK96296.1	Nitroreductase family	Eubacterium siraeum 70/3	38.9	159	6.0E-45
167750792	ZP_02422919.1	hypothetical protein EUBSIR_01774	Eubacterium siraeum DSM 15702	39.3	159	7.0E-45
374624294	ZP_09696711.1	hypothetical protein HMPREF0978_00031	Coprobacillus sp. 8_2_54BFAA	38.5	159	1.0E-44
167756101	ZP_02428228.1	hypothetical protein CLORAM_01621	Clostridium ramosum DSM 1402	38.5	158	2.0E-44
291557779	CBL34896.1	Nitroreductase family	Eubacterium siraeum V10Sc8a	38.4	158	2.0E-44
237734085	ZP_04564566.1	nitroreductase	Mollicutes bacterium D7	38.5	158	2.0E-44
302336278	YP_003801485.1	nitroreductase	Olsenella uli DSM 7084	40.9	157	3.0E-44
365133438	ZP_09342773.1	hypothetical protein HMPREF1032_00569	Subdoligranulum sp. 4_3_54A2FAA	38.9	157	3.0E-44
255280695	ZP_05345250.1	nitroreductase family protein	Bryantella formatexigens DSM 14469	35.1	156	1.0E-43

Appendix 9

BLASTP results of g1083, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value ²
281421596	ZP_06252595.1	nitroreductase family protein	Prevotella copri DSM 18205	35.5	154	9.0E-43
320528159	ZP_08029324.1	nitroreductase family protein	Solobacterium moorei F0204	38.5	154	1.0E-42
260439137	ZP_05792953.1	nitroreductase family protein	Butyrivibrio crossotus DSM 2876	36.4	152	2.0E-42
256827090	YP_003151049.1	nitroreductase	Cryptobacterium curtum DSM 15641	34.6	151	1.0E-41
373494580	ZP_09585183.1	hypothetical protein HMPREF0380_00821	Eubacterium infirmum F0142	36.9	150	2.0E-41
163815754	ZP_02207126.1	hypothetical protein COPEUT_01935	Coprococcus eutactus ATCC 27759	36.4	150	3.0E-41
300727300	ZP_07060714.1	nitroreductase family protein	Prevotella bryantii B14	36.0	148	2.0E-40
300727386	ZP_07060800.1	nitroreductase family protein	Prevotella bryantii B14	36.1	147	2.0E-40
295099618	CBK88707.1	hypothetical protein	Eubacterium cylindroides T2-87	37.1	147	3.0E-40
261415319	YP_003249002.1	nitroreductase	Fibrobacter succinogenes subsp. succinogenes S85	39.1	146	1.0E-39
319938164	ZP_08012562.1	nitroreductase	Coprobacillus sp. 29_1	36.0	143	1.0E-38
330836540	YP_004411181.1	unnamed protein product	Spirochaeta coccoides DSM 17374	35.5	138	1.0E-36
269216833	ZP_06160687.1	Nitroreductase family protein	Slackia exigua ATCC 700122	37.7	135	9.0E-36
303234622	ZP_07321256.1	conserved hypothetical protein	Finegoldia magna BVS033A4	31.2	130	1.0E-33
169825211	YP_001692822.1	putative nitroreductase	Finegoldia magna ATCC 29328	31.6	129	3.0E-33
302380466	ZP_07268934.1	conserved hypothetical protein	Finegoldia magna ACS-171-V-Col3	32.0	128	5.0E-33
343521061	ZP_08758029.1	nitroreductase domain protein	Parvimonas sp. oral taxon 393 str. F0440	32.3	128	5.0E-33
341592162	EGS35048.1	hypothetical protein HMPREF9489_1771	Finegoldia magna SY403409CC001050417	30.7	128	7.0E-33
340359493	ZP_08681978.1	hypothetical protein HMPREF9062_1103	Actinomyces sp. oral taxon 448 str. F0400	37.6	127	9.0E-33

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BLASTP results of g1083, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Sources	% Identity	Score	E-value ²
366164402	ZP_09464157.1	nitroreductase family protein	Acetivibrio cellulolyticus CD2	34.2	125	8.0E-32
239627032	ZP_04670063.1	sensor histidine kinase/response regulator	Clostridiales bacterium 1_7_47_FAA	35.3	124	3.0E-31
342215982	ZP_08708629.1	nitroreductase domain protein	Peptoniphilus sp. oral taxon 375 str. F0436	36.4	124	3.0E-31
355574191	ZP_09044034.1	hypothetical protein HMPREF1008_00011	Olsenella sp. oral taxon 809 str. F0356	36.0	126	3.0E-31
304383237	ZP_07365709.1	conserved hypothetical protein	Prevotella marshii DSM 16973	33.3	122	9.0E-31
160939059	ZP_02086410.1	hypothetical protein CLOBOL_03953	Clostridium bolteae ATCC BAA-613	33.6	122	2.0E-30
374308483	YP_005054914.1	unnamed protein product	Filifactor alocis ATCC 35896	35.0	122	3.0E-30
294790995	ZP_06756153.1	putative Nitroreductase family protein	Scardovia inopinata F0304	35.6	120	4.0E-30
306823919	ZP_07457293.1	conserved hypothetical protein	Bifidobacterium dentium ATCC 27679	37.2	120	4.0E-30
171741656	ZP_02917463.1	hypothetical protein BIFDEN_00742	Bifidobacterium dentium ATCC 27678	37.2	120	5.0E-30
297587905	ZP_06946549.1	probable nitroreductase	Finegoldia magna ATCC 53516	30.3	120	5.0E-30
313900802	ZP_07834292.1	conserved hypothetical protein	Clostridium sp. HGF2	33.9	119	2.0E-29
315926066	ZP_07922266.1	nitroreductase	Pseudoramibacter alactolyticus ATCC 23263	33.9	118	6.0E-29
346314935	ZP_08856452.1	hypothetical protein HMPREF9022_02109	Erysipelotrichaceae bacterium 2_2_44A	34.6	117	7.0E-29
227872464	ZP_03990805.1	nitroreductase family protein	Oribacterium sinus F0268	34.5	117	9.0E-29
218282774	ZP_03488956.1	hypothetical protein EUBIFOR_01542	Eubacterium bifforme DSM 3989	32.5	116	1.0E-28
169640151	ACA61155.1	nitroreductase family protein, partial	uncultured microorganism	31.1	115	4.0E-28
306819723	ZP_07453383.1	nitroreductase	Eubacterium yurii subsp. margaretae ATCC 43715	33.8	114	2.0E-27
335045920	ZP_08538943.1	hypothetical protein	Oribacterium sp. oral taxon 108 str.	34.2	112	6.0E-27

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BLASTP results of g1083, a potential nitroreductase expressed by *Bifidobacterium breve* M-16V (list of top 100)¹

Query (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value
		HMPREF9124_1362	F0425			
229827999	ZP_04454068.1	hypothetical protein GCWU000342_00048	Shuttleworthia satelles DSM 14600	31.4	112	8.0E-27
357053084	ZP_09114187.1	hypothetical protein HMPREF9467_01159	Clostridium clostridioforme 2_1_49FAA	32.8	111	1.0E-26
363897349	ZP_09323888.1	hypothetical protein HMPREF9624_00450	Oribacterium sp. ACB7	33.6	111	2.0E-26
309775082	ZP_07670095.1	nitroreductase family protein	Erysipelotrichaceae bacterium 3_1_53	33.9	111	2.0E-26
283768792	ZP_06341703.1	conserved hypothetical protein	Bulleidia extracta W1219	34.0	111	3.0E-26
363899320	ZP_09325830.1	hypothetical protein HMPREF9625_00490	Oribacterium sp. ACB1	33.2	109	6.0E-26
363899923	ZP_09326429.1	hypothetical protein HMPREF9625_01089	Oribacterium sp. ACB1	36.1	109	2.0E-25
323340072	ZP_08080338.1	hypothetical protein HMPREF0542_10769	Lactobacillus ruminis ATCC 25644	32.2	106	8.0E-25
387133375	YP_006299347.1	hypothetical protein PIN17_A1468	Prevotella intermedia 17	30.3	106	9.0E-25
167747743	ZP_02419870.1	hypothetical protein ANACAC_02464	Anaerostipes caccae DSM 14662	34.8	105	3.0E-24
317473480	ZP_07932772.1	hypothetical protein HMPREF1011_03122	Anaerostipes sp. 3_2_56FAA	34.8	104	9.0E-24
23464674	NP_695277.1	hypothetical protein BL0046	Bifidobacterium longum NCC2705	30.1	104	9.0E-24
46190638	ZP_00121302.2	COG0778: Nitroreductase	Bifidobacterium longum DJO10A	30.1	104	1.0E-23
227546775	ZP_03976824.1	nitroreductase	Bifidobacterium longum subsp. infantis ATCC 55813	30.1	104	1.0E-23
384201097	YP_005586844.1	unnamed protein product	Bifidobacterium longum subsp. longum KACC 91563	30.1	103	2.0E-23
294786742	ZP_06751996.1	nitroreductase family protein	Parascardovia denticolens F0305	31.3	103	3.0E-23
15894035	NP_347384.1	unnamed protein product	Clostridium acetobutylicum ATCC 824	30.5	101	8.0E-23

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BLASTP results of g1083, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Sources	% Identity	Score	E-value ²
229826652	ZP_04452721.1	hypothetical protein GCWU000182_02028	Abiotrophia defectiva ATCC 49176	32.0	100	2.0E-22
363898321	ZP_09324855.1	hypothetical protein HMPREF9624_01417	Oribacterium sp. ACB7	35.9	100	2.0E-22
325840230	ZP_08166997.1	hypothetical protein HMPREF9402_1542	Turicibacter sp. HGF1	32.5	99.8	4.0E-22
335045198	ZP_08538221.1	hypothetical protein HMPREF9124_2268	Oribacterium sp. oral taxon 108 str. F0425	35.5	99.8	4.0E-22
293375510	ZP_06621787.1	conserved hypothetical protein	Turicibacter sanguinis PC909	32.5	99.4	6.0E-22
291457490	ZP_06596880.1	conserved hypothetical protein	Bifidobacterium breve DSM 20213 = JCM 1192	30.7	98.6	1.0E-21
302392258	YP_003828078.1	unnamed protein product	Acetohalobium arabaticum DSM 5501	30.3	98.6	2.0E-21
384196688	YP_005582432.1	unnamed protein product	Bifidobacterium breve ACS-071-V-Sch8b	30.8	98.2	2.0E-21
374316643	YP_005063071.1	unnamed protein product	Sphaerochaeta pleomorpha str. Grapes	31.1	97.1	4.0E-21
373107128	ZP_09521428.1	hypothetical protein HMPREF9623_01092	Lachnospiraceae bacterium ACC2	32.4	96.3	6.0E-21
193216521	YP_001999763.1	nitroreductase family protein	Mycoplasma arthritidis 158L3-1	28.5	94.4	3.0E-20
297204981	ZP_06922377.1	possible nitroreductase	Lactobacillus jensenii JV-V16	31.2	93.2	7.0E-20
256852140	ZP_05557527.1	nitroreductase	Lactobacillus jensenii 27-2-CHN	31.2	93.2	8.0E-20
227873788	ZP_03992015.1	nitroreductase family protein	Oribacterium sinus F0268	31.7	93.2	9.0E-20
257064989	YP_003144661.1	nitroreductase	Slackia heliotrinireducens DSM 20476	32.1	90.5	9.0E-19
257065108	YP_003144780.1	nitroreductase	Slackia heliotrinireducens DSM 20476	31.9	87.8	7.0E-18
160879506	YP_001558474.1	hypothetical protein Cphy_1360	Clostridium phytofermentans ISDg	29.7	87.4	9.0E-18
354558568	ZP_08977823.1	nitroreductase	Desulfitobacterium metallireducens DSM 15288	26.5	87	2.0E-17

Appendix 9

BLASTP results of g1083, a potential nitroreductase expressed by *Bifidobacterium breve* M-16V (list of top 100)¹

Query (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value ²
89893795	YP_517282.1	hypothetical protein DSY1049	Desulfitobacterium hafniense Y51	30.7	85.5	4.0E-17
374381426	ZP_09639015.1	nitroreductase	Desulfitobacterium dichloroeliminans LMG P-21439	27.7	85.1	8.0E-17
225182034	ZP_03735465.1	nitroreductase	Dethiobacter alkaliphilus AHT 1	30.0	84.7	8.0E-17
219668168	YP_002458603.1	nitroreductase	Desulfitobacterium hafniense DCB-2	30.1	83.2	2.0E-16
260664952	ZP_05865803.1	nitroreductase	Lactobacillus jensenii SJ-7A-US	30.0	79	5.0E-15
238855120	ZP_04645446.1	putative nitroreductase	Lactobacillus jensenii 269-3	30.0	79	5.0E-15
295106735	CBL04278.1	hypothetical protein	Gordonibacter pamelaee 7-10-1-b	37.0	77	2.0E-14
221194690	ZP_03567747.1	nitroreductase family protein	Atopobium rimae ATCC 49626	29.2	77	3.0E-14
34392796	BAC82711.1	putative nitroreductase	Fingoldia magna ATCC 29328	32.1	74.3	5.0E-14
257784997	YP_003180214.1	nitroreductase	Atopobium parvulum DSM 20469	29.2	76.3	7.0E-14

¹The open reading frame of g1038 was determined as described {Kosuge et al., 2006, DNA Res, 13, 245-54} and BLASTPed against the NCBI data base (nr, 20120604).
²E-value of 0 to 1E-100 equals high homology; E-value of 1E-100 to 1E-50 equals medium homology; E-value of 1E-50 to 1E-30 equals low homology; E-values of >1E-30 equals no homology.

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BLASTP results of g1858, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value ²
291456953	ZP_06596343.1	nitro/flavin reductase	Bifidobacterium breve DSM 20213	100.0	522	0.0E+00
376167384	EHS86228.1	Nitro/flavin reductase	Bifidobacterium breve CECT 7263	98.8	514	0.0E+00
296455085	YP_003662229.1	nitroreductase	[Bifidobacterium longum subsp. longum JDM301	84.7	453	3.0E-159
227546878	ZP_03976927.1	flavin reductase	Bifidobacterium longum subsp. infantis ATCC 55813	84.3	451	2.0E-158
384200523	YP_005586266.1	unnamed protein product	Bifidobacterium longum subsp. infantis ATCC 15697	84.7	452	3.0E-158
213693293	YP_002323879.1	nitroreductase	Bifidobacterium longum subsp. infantis ATCC 15697	84.7	451	3.0E-158
312133694	YP_004001033.1	nfnb2	Bifidobacterium longum subsp. longum BBMN68	83.9	449	8.0E-158
46190575	ZP_00121309.2	Nitroreductase	Bifidobacterium longum DJO10A	84.7	449	8.0E-158
386408403	EIJ23314.1	nitroreductase family protein	Bifidobacterium longum subsp. longum 1-6B	84.3	451	9.0E-158
317482619	ZP_07941633.1	nitroreductase	Bifidobacterium sp. 12_1_47BFAA	83.9	449	1.0E-157
291516640	CBK70256.1	Nitroreductase	Bifidobacterium longum subsp. longum F8	83.9	449	1.0E-157
386411477	EIJ26204.1	nitroreductase family protein	Bifidobacterium longum subsp. longum 35B	83.9	448	2.0E-157
23336774	ZP_00121951.1	Nitroreductase	Bifidobacterium longum DJO10A	85.3	405	1.0E-140
309801831	ZP_07695949.1	nitroreductase family protein	Bifidobacterium dentium JCVIHMP022	75.9	396	7.0E-137
171741760	ZP_02917567.1	hypothetical protein	Bifidobacterium dentium ATCC 27678	75.9	396	1.0E-136
306824067	ZP_07457439.1	nitro/flavin reductase	Bifidobacterium dentium ATCC 27679	75.9	396	1.0E-136
212716572	ZP_03324700.1	hypothetical protein	Bifidobacterium catenulatum DSM 16992	74.7	390	4.0E-134
154489183	ZP_02030032.1	hypothetical protein	Bifidobacterium adolescentis L2-32	73.9	387	3.0E-133

Appendix 9

BLASTP results of g1858, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value
225352739	ZP_03743762.1	hypothetical protein	<i>Bifidobacterium pseudocatenulatum</i> DSM 20438	73.9	387	3.0E-133
119026586	YP_910431.1	NADPH-flavin oxidoreductase	<i>Bifidobacterium adolescentis</i> ATCC 15703	73.9	387	4.0E-133
311065128	YP_003971854.1	chromate reductase/NADPH-dependent FMN reductase/Oxygen-insensitive NADPH nitroreductase	<i>Bifidobacterium bifidum</i> PRL2010	70.3	375	2.0E-128
313140626	ZP_07802819.1	nitroreductase	<i>Bifidobacterium bifidum</i> NCIMB 41171	70.3	375	2.0E-128
310288265	YP_003939524.1	Nitroreductase family protein	<i>Bifidobacterium bifidum</i> S17	69.9		5.0E-127
229817025	ZP_04447307.1	hypothetical protein BIFANG_02280	<i>Bifidobacterium angulatum</i> DSM 20098 = JCM 7096	70.0	372	5.0E-122
298252499	ZP_06976294.1	nitroreductase	<i>Gardnerella vaginalis</i> 5-1	68.0	359	2.0E-121
283783743	YP_003374497.1	nitroreductase family protein	<i>Gardnerella vaginalis</i> 409-05	67.6	357	7.0E-121
297242635	ZP_06926574.1	nitroreductase	<i>Gardnerella vaginalis</i> AMD	66.0	357	5.0E-118
23464759	NP_695362.1	NADPH-flavin oxidoreductase; flavin reductase P; NADPH-FMN oxidoreductase	<i>Bifidobacterium longum</i> NCC2705	88.0	349	1.0E-114
385801086	YP_005837489.1	unnamed protein product	<i>Gardnerella vaginalis</i> HMP9231	63.5	337	4.0E-113
311114115	YP_003985336.1	nitroreductase	<i>Gardnerella vaginalis</i> ATCC 14019	63.1	337	3.0E-112
333602053	EGL13485.1	nitroreductase family protein	<i>Gardnerella vaginalis</i> 315-A	63.1	334	3.0E-112
261338723	ZP_05966607.1	nitro/flavin reductase	<i>Bifidobacterium gallicum</i> DSM 20093	61.7	334	4.0E-103
183602925	ZP_02964281.1	possible NADPH-flavin oxidoreductase	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> HN019	56.4	311	4.0E-93
386867687	YP_006280681.1	NADPH-flavin oxidoreductase	<i>Bifidobacterium animalis</i> subsp. <i>animalis</i> ATCC 25527	55.2	285	3.0E-91
384200579	YP_005586326.1	unnamed protein product	<i>Bifidobacterium longum</i> subsp. <i>longum</i> KACC 91563	80.9	280	8.0E-72

BLASTP results of g1858, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Sources	% Identity	Score	E-value ²
374711191	ZP_09715625.1	NADPH-dependent oxidoreductase	Sporolactobacillus inulinus CASD	47.6	226	6.0E-64
259047355	ZP_05737756.1	nitro/flavin reductase	Granulicatella adiacens ATCC 49175	43.5	210	6.0E-62
336393046	ZP_08574445.1	nitro/flavin reductase	Lactobacillus coryniformis subsp. torquens KCTC 3535	45.5	205	1.0E-61
333394407	ZP_08476226.1	nitro/flavin reductase	Lactobacillus coryniformis subsp. coryniformis KCTC 3167	45.5	204	3.0E-61
340398920	YP_004727945.1	NADPH-dependent oxidoreductase	Streptococcus salivarius CCHSS3	44.4	203	1.0E-60
323343158	ZP_08083389.1	oxygen-insensitive NADPH nitroreductase	Erysipelothrix rhusiopathiae ATCC 19414	43.7	202	1.0E-60
228477406	ZP_04062042.1	NADPH-dependent oxidoreductase	Streptococcus salivarius SK126	44.8	202	2.0E-60
387761389	YP_006068366.1	putative NADPH-dependent oxidoreductase	Streptococcus salivarius 57.I	44.4	201	4.0E-60
336065394	YP_004560252.1	unnamed protein product	Erysipelothrix rhusiopathiae str. Fujisawa	43.7	200	7.0E-60
345526748	EGX30059.1	NADPH-dependent oxidoreductase	Streptococcus salivarius M18	44.4	200	1.0E-59
322516701	ZP_08069610.1	NADPH-dependent oxidoreductase	Streptococcus vestibularis ATCC 49124	44.1	199	2.0E-59
387784067	YP_006070150.1	NADPH-dependent oxidoreductase	Streptococcus salivarius JIM8777	44.4	199	2.0E-59
312862901	ZP_07723141.1	putative NADPH-dependent oxidoreductase	Streptococcus vestibularis F0396	44.1	198	4.0E-59
322372862	ZP_08047398.1	nitroreductase family protein	Streptococcus sp. C150	44.2	198	4.0E-59
55821160	YP_139602.1	nitro/flavin reductase	Streptococcus thermophilus LMG 18311	44.4	197	5.0E-59
116627876	YP_820495.1	nitro/flavin reductase	Streptococcus thermophilus LMD-9	44.1	197	2.0E-58
383282481	EIC80467.1	Nitro/flavin reductase	Streptococcus salivarius PS4	44.2	196	2.0E-58
335358167	ZP_08550037.1	nitroreductase	Lactobacillus animalis KCTC 3501	42.7	196	1.0E-57

BLASTP results of g1858, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
Query (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value
269976097	ZP_06183096.1	oxygen-insensitive NADPH nitroreductase	Mobiluncus mulieris 28-1	44.8	194	1.0E-57
259502227	ZP_05745129.1	oxygen-insensitive NADPH nitroreductase	Lactobacillus antri DSM 16041	44.5	194	1.0E-57
312870362	ZP_07730487.1	putative oxygen-insensitive NADPH nitroreductase	Lactobacillus oris PB013-T2-3	44.5	194	4.0E-57
289423241	ZP_06425052.1	oxygen-insensitive NADPH nitroreductase	Peptostreptococcus anaerobius 653-L	43.4	193	4.0E-57
326803084	YP_004320902.1	nitroreductase family protein	Aerococcus urinae ACS-120-V-Col10a	42.2	196	5.0E-57
227876327	ZP_03994440.1	possible flavin reductase	Mobiluncus mulieris ATCC 35243	44.6	192	7.0E-57
347524716	YP_004831464.1	Nitroreductase	Lactobacillus ruminis ATCC 27782	43.9	192	2.0E-56
323341538	ZP_08081776.1	oxygen-insensitive NADPH nitroreductase	Lactobacillus ruminis ATCC 25644	43.9	191	1.0E-55
306817218	ZP_07450965.1	oxygen-insensitive NADPH nitroreductase	Mobiluncus mulieris ATCC 35239	44.2	189	1.0E-55
375292157	YP_005126696.1	nfrA gene product	Corynebacterium diphtheriae INCA 402	44.3	189	2.0E-55
297571519	YP_003697293.1	nitroreductase	Arcanobacterium haemolyticum DSM 20595	40.8	189	2.0E-55
363892306	ZP_09319474.1	hypothetical protein HMPREF9630_00467	Eubacteriaceae bacterium CM2	37.7	188	5.0E-55
363893758	ZP_09320853.1	hypothetical protein HMPREF9629_01179	Eubacteriaceae bacterium ACC19a	37.3	187	6.0E-55
376247549	YP_005139493.1	nfrA gene product	Corynebacterium diphtheriae HC04	43.8	187	9.0E-55
363889009	ZP_09316376.1	hypothetical protein HMPREF9628_01012	Eubacteriaceae bacterium CM5	37.3	187	9.0E-55
376256184	YP_005144075.1	unnamed protein product	Corynebacterium diphtheriae VA01	43.8	186	1.0E-54
376241919	YP_005132771.1	unnamed protein product	Corynebacterium diphtheriae CDCE 8392	43.8	186	1.0E-54

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BLASTP results of g1858, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100) ¹						
GenInfo (GI) Number	Gene Accession Number	Definition	Source	% Identity	Score	E-value ²
38232888	NP_938655.1	nitroreductase	Corynebacterium diphtheriae NCTC 13129	43.8	187	1.0E-54
376253389	YP_005141848.1	unnamed protein product	Corynebacterium diphtheriae PW8	43.8	186	3.0E-54
376250366	YP_005137247.1	nfrA gene product	Corynebacterium diphtheriae HC03	43.4	186	3.0E-54
377831755	ZP_09814725.1	flavin reductase	Lactobacillus mucosae LM1	42.8	186	4.0E-54
194467040	ZP_03073027.1	nitroreductase	Lactobacillus reuteri 100-23	43.2	185	5.0E-54
376286744	YP_005159310.1	nfrA gene product	Corynebacterium diphtheriae BH8	43.4	184	8.0E-54
376289421	YP_005161668.1	unnamed protein product	Corynebacterium diphtheriae C7 (beta)	43.4	184	1.0E-53
227496850	ZP_03927116.1	possible flavin reductase	Actinomyces urogenitalis DSM 15434	43.3	184	1.0E-53
227545017	ZP_03975066.1	flavin reductase	Lactobacillus reuteri CF48-3A	43.2	184	2.0E-53
340359089	ZP_08681585.1	oxygen-insensitive NADPH nitroreductase	Actinomyces sp. oral taxon 448 str. F0400	43.9	183	4.0E-53
342214762	ZP_08707436.1	nitroreductase family protein	Veillonella sp. oral taxon 780 str. F0422	41.0	183	6.0E-53
184154650	YP_001842990.1	nitroreductase	Lactobacillus fermentum IFO 3956	40.6	182	6.0E-53
227529750	ZP_03959799.1	flavin reductase	Lactobacillus vaginalis ATCC 49540	39.5	182	9.0E-53
325479472	EGC82568.1	putative nitro/flavin reductase	Anaerococcus prevotii ACS-065-V-Col13	40.2	181	1.0E-52
68536654	YP_251359.1	oxygen-insensitive NADPH nitroreductase	Corynebacterium jeikeium K411	41.4	181	1.0E-52
337728358	CCC03458.1	nitroreductase	Lactobacillus reuteri ATCC 53608	42.4	181	2.0E-52
260577854	ZP_05845787.1	nitro/flavin reductase	Corynebacterium jeikeium ATCC 43734	41.7	181	4.0E-52
90962348	YP_536264.1	nitro/flavin reductase	Lactobacillus salivarius UCC118	40.4	179	4.0E-52
260584649	ZP_05852395.1	nitro/flavin reductase	Granulicatella elegans ATCC 700633	41.1	179	1.0E-51

Appendix 9

BLASTP results of g1858, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100)¹						
Contig (GI) Number	Gene Accession Number	Description	Source	% Identity	Score	E-value
148544770	YP_001272140.1	nitroreductase	Lactobacillus reuteri DSM 20016	41.9	178	2.0E-51
295692342	YP_003600952.1	nitro/flavin reductase	Lactobacillus crispatus ST1	41.0	178	2.0E-51
227891257	ZP_04009062.1	flavin reductase	Lactobacillus salivarius ATCC 11741	38.7	178	3.0E-51
365852105	ZP_09392508.1	putative oxygen-insensitive NADPH nitroreductase	Lactobacillus parafarraginis F0439	42.0	177	3.0E-51
227891581	ZP_04009386.1	flavin reductase	Lactobacillus salivarius ATCC 11741	40.0	177	4.0E-51
257066489	YP_003152745.1	nitroreductase	Anaerococcus prevotii DSM 20548	40.8	177	5.0E-51
301300706	ZP_07206892.1	nitroreductase family protein	Lactobacillus salivarius ACS-116-V-Col5a	40.0	177	1.0E-50
298345584	YP_003718271.1	putative nitroreductase	Mobiluncus curtisii ATCC 43063	41.0	176	1.0E-50
375090877	ZP_09737184.1	hypothetical protein HMPREF9709_00046	Helcococcus kunzii ATCC 51366	37.8	176	1.0E-50
377556884	ZP_09786561.1	Nitroreductase	Lactobacillus gastricus PS3	38.3	176	2.0E-50
365924696	ZP_09447459.1	Nitroreductase	Lactobacillus mali KCTC 3596 = DSM 20444	38.4	176	2.0E-50

¹The open reading frame of g1858 was determined as described {Kosuge et al., 2006, DNA Res, 13, 245-54} and BLASTPed against the NCBI data base (nr, 20120604).
²E-value of 0 to 1E-100 equals high homology; E-value of 1E-100 to 1E-50 equals medium homology; E-value of 1E-50 to 1E-30 equals low homology; E-values of >1E-30 equals no homology.

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Appendix 10

BLASTP ¹ results of g0647, a potential nitroreductase expressed by <i>Bifidobacterium breve</i> M-16V (list of top 100).						
GenInfo (GI) Number	Gene Accession Number	Definition	Sources	% Identity	Score	E-value ²
291456443	ZP_06595833.1	ribosomal RNA large subunit methyltransferase J	Bifidobacterium breve DSM 20213	100.0	499	8.0E-178
376168127	EHS86935.1	Ribosomal RNA large subunit methyltransferase J	Bifidobacterium breve CECT 7263	99.2	494	1.0E-175
384197242	YP_005582986.1	rrmJ gene product	Bifidobacterium breve ACS-071-V-Sch8b	98.8	492	1.0E-174
339478932	ABE95393.1	Hemolysin-like protein with S4 and FtsJ-like methyltransferase domains	Bifidobacterium breve UCC2003	98.4	489	2.0E-173
384199936	YP_005585679.1	unnamed protein product	Bifidobacterium longum subsp. infantis ATCC 15697	88.7	439	1.0E-153
322688777	YP_004208511.1	hypothetical protein BLIF_0590	Bifidobacterium longum subsp. infantis 157F	88.7	438	2.0E-153
296453816	YP_003660959.1	hemolysin A	Bifidobacterium longum subsp. longum JDM301	88.7	437	3.0E-153
213692730	YP_002323316.1	hemolysin A	Bifidobacterium longum subsp. infantis ATCC 15697	88.7	438	4.0E-153
227546214	ZP_03976263.1	hemolysin A	Bifidobacterium longum subsp. infantis ATCC 55813	88.3	436	2.0E-152
23465615	NP_696218.1	hemolysin-like protein	Bifidobacterium longum NCC2705	88.3	434	1.0E-151
46191091	ZP_00206673.1	COG1189: Predicted rRNA methylase	Bifidobacterium longum DJO10A	87.9	434	1.0E-151
229817935	ZP_04448217.1	hypothetical protein BIFANG_03222	Bifidobacterium angulatum DSM 20098	73.6	357	4.0E-121
313140389	ZP_07802582.1	conserved hypothetical protein	Bifidobacterium bifidum NCIMB 41171	71.6	331	2.0E-111
310287601	YP_003938859.1	FtsJ-like methyltransferase domain	Bifidobacterium bifidum S17	71.2	332	2.0E-111
119025929	YP_909774.1	hemolysin-like protein	Bifidobacterium adolescentis ATCC 15703	70.3	330	8.0E-111
225352189	ZP_03743212.1	hypothetical protein BIFPSEUDO_03805	Bifidobacterium pseudocatenulatum DSM 20438	69.7	327	8.0E-110

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212715757	ZP_03323885.1	hypothetical protein BIFCAT_00657	<i>Bifidobacterium catenulatum</i> DSM 16992	69.7	324	2.0E-108
154488635	ZP_02029484.1	hypothetical protein BIFADO_01942	<i>Bifidobacterium adolescentis</i> L2-32	70.9	320	4.0E-107
261337851	ZP_05965735.1	cytotoxin/hemolysin	<i>Bifidobacterium gallicum</i> DSM 20093	70.0	321	8.0E-107
171742791	ZP_02918598.1	hypothetical protein BIFDEN_01905	<i>Bifidobacterium dentium</i> ATCC 27678	68.2	318	4.0E-106
306822649	ZP_07456027.1	ribosomal RNA large subunit methyltransferase J	<i>Bifidobacterium dentium</i> ATCC 27679	68.2	317	5.0E-106
241190773	YP_002968167.1	hemolysin-like protein	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> BI-04	67.1	292	3.0E-96
183601729	ZP_02963099.1	possible rRNA methyltransferase	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> HN019	67.1	293	3.0E-96
219683739	YP_002470122.1	hemolysin-like protein	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> AD011	67.1	293	4.0E-96
283783153	YP_003373907.1	ribosomal RNA large subunit methyltransferase J	<i>Gardnerella vaginalis</i> 409-05	59.2	292	6.0E-96
298253921	ZP_06977508.1	rRNA methylase	<i>Gardnerella vaginalis</i> 5-1	59.2	290	6.0E-95
297243603	ZP_06927534.1	rRNA methylase	<i>Gardnerella vaginalis</i> AMD	58.2	289	1.0E-94
385801884	YP_005838287.1	rrmJ gene product	<i>Gardnerella vaginalis</i> HMP9231	59.9	286	1.0E-93
308235815	ZP_07666552.1	ribosomal RNA large subunit methyltransferase J	<i>Gardnerella vaginalis</i> ATCC 14018 = JCM 11026	59.9	286	2.0E-93
333602935	EGL14360.1	ribosomal RNA large subunit methyltransferase J	<i>Gardnerella vaginalis</i> 315-A	59.5	283	2.0E-92
294787547	ZP_06752800.1	ribosomal RNA large subunit methyltransferase J	<i>Parascardovia denticolens</i> F0305	53.6	245	1.0E-77
283456159	YP_003360723.1	hemolysin-like protein	<i>Bifidobacterium dentium</i> Bd1	74.0	217	4.0E-68
294791518	ZP_06756675.1	cytotoxin/hemolysin	<i>Scardovia inopinata</i> F0304	47.7	218	8.0E-67
296269986	YP_003652618.1	hemolysin A	<i>Thermobispora bispora</i> DSM 43833	49.6	204	2.0E-61
269126403	YP_003299773.1	hemolysin A	<i>Thermomonospora curvata</i> DSM 43183	49.0	203	4.0E-61

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271967338	YP_003341534.1	unnamed protein product	<i>Streptosporangium roseum</i> DSM 43021	47.9	202	1.0E-60
375096125	ZP_09742390.1	hemolysin A	<i>Saccharomonospora marina</i> XMU15	48.8	198	3.0E-59
154251238	YP_001412062.1	unnamed protein product	<i>Parvibaculum lavamentivorans</i> DS-1	46.7	197	3.0E-59
148273167	YP_001222728.1	hypothetical protein CMM_1986	<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> NCPPB 382	50.2	199	5.0E-59
170781652	YP_001709984.1	hypothetical protein CMS_1246	<i>Clavibacter michiganensis</i> subsp. <i>sepedonicus</i>	51.0	197	1.0E-58
377571060	ZP_09800185.1	hemolysin	<i>Gordonia terrae</i> NBRC 100016	46.7	196	4.0E-58
227821073	YP_002825043.1	hemolysin-like protein	<i>Sinorhizobium fredii</i> NGR234	47.7	196	5.0E-58
300787880	YP_003768171.1	hemolysin-like protein	<i>Amycolatopsis mediterranei</i> U32	47.3	194	5.0E-58
269218361	ZP_06162215.1	cytotoxin/hemolysin	<i>Actinomyces</i> sp. oral taxon 848 str. F0332	46.5	195	5.0E-58
254385883	ZP_05001202.1	rRNA methylase	<i>Streptomyces</i> sp. Mg1	47.7	195	6.0E-58
78043160	YP_360803.1	unnamed protein product	<i>Carboxydotherrmus hydrogenoformans</i> Z-2901	45.0	195	7.0E-58
219847267	YP_002461700.1	hemolysin A	<i>Chloroflexus aggregans</i> DSM 9485	49.0	194	8.0E-58
150395716	YP_001326183.1	unnamed protein product	<i>Sinorhizobium medicae</i> WSM419	47.8	194	8.0E-58
347755549	YP_004863113.1	unnamed protein product	<i>Candidatus Chloracidobacterium thermophilum</i> B	47.7	194	1.0E-57
163849205	YP_001637249.1	hemolysin A	<i>Chloroflexus aurantiacus</i> J-10-fl	46.7	194	1.0E-57
308176937	YP_003916343.1	haemolysin	<i>Arthrobacter arilaitensis</i> Re117	45.3	194	1.0E-57
313679575	YP_004057314.1	unnamed protein product	<i>Oceanithermus profundus</i> DSM 14977	49.0	193	2.0E-57
365824293	ZP_09366367.1	hypothetical protein HMPREF0045_00003	<i>Actinomyces graevenitzii</i> C83	48.0	193	2.0E-57
378825150	YP_005187882.1	unnamed protein product	<i>Sinorhizobium fredii</i> HH103	46.9	194	2.0E-57
344998819	YP_004801673.1	hemolysin A	<i>Streptomyces</i> sp. SirexAA-E	47.3	192	8.0E-57
327188063	EGE55290.1	hemolysin A	<i>Rhizobium etli</i> CNPAF512	46.3	192	9.0E-57

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72162433	YP_290090.1	unnamed protein product	<i>Thermobifida fusca</i> YX	49.6	191	2.0E-56
357398588	YP_004910513.1	yqxC gene product	<i>Streptomyces cattleya</i> NRRL 8057 = DSM 46488	47.8	191	2.0E-56
284033301	YP_003383232.1	hemolysin A	<i>Kribbella flavida</i> DSM 17836	46.9	191	2.0E-56
378549324	ZP_09824540.1	hypothetical protein CCH26_04525	<i>Citricoccus</i> sp. CH26A	47.3	191	3.0E-56
359501478	EHK74086.1	hemolysin A	<i>Sinorhizobium meliloti</i> CCNWSX0020	46.1	190	3.0E-56
195970192	NP_384985.2	hypothetical protein SMc00973	<i>Sinorhizobium meliloti</i> 1021	46.1	191	3.0E-56
377565546	ZP_09794836.1	hemolysin	<i>Gordonia sputi</i> NBRC 100414	45.9	190	3.0E-56
167766571	ZP_02438624.1	hypothetical protein CLOSS21_01077	<i>Clostridium</i> sp. SS2/1	42.2	190	4.0E-56
384566694	ZP_10013798.1	hemolysin A	<i>Saccharomonospora glauca</i> K62	46.7	190	5.0E-56
240170143	ZP_04748802.1	hypothetical protein MkanA1_12573	<i>Mycobacterium kansasii</i> ATCC 12478	46.1	190	6.0E-56
262202727	YP_003273935.1	unnamed protein product	<i>Gordonia bronchialis</i> DSM 43247	46.5	191	7.0E-56
158313587	YP_001506095.1	unnamed protein product	<i>Frankia</i> sp. EAN1pec	46.3	190	7.0E-56
227494617	ZP_03924933.1	hemolysin A	<i>Actinomyces coleocanis</i> DSM 15436	46.5	190	8.0E-56
297571513	YP_003697287.1	hemolysin A	<i>Arcanobacterium haemolyticum</i> DSM 20595	45.3	191	8.0E-56
121534494	ZP_01666317.1	hemolysin A	<i>Thermosinus carboxydivorans</i> Nor1	47.2	189	1.0E-55
218462484	ZP_03502575.1	hemolysin A	<i>Rhizobium etli</i> Kim 5	45.6	189	1.0E-55
51892979	YP_075670.1	rRNA methylase	<i>Symbiobacterium thermophilum</i> IAM 14863	47.6	189	2.0E-55
365863474	ZP_09403189.1	putative rRNA methylase	<i>Streptomyces</i> sp. W007	47.4	189	2.0E-55
357414216	YP_004925952.1	hemolysin A	<i>Streptomyces flavogriseus</i> ATCC 33331	46.9	189	2.0E-55
363421746	ZP_09309829.1	hemolysin	<i>Rhodococcus pyridinivorans</i> AK37	45.3	189	2.0E-55
227549334	ZP_03979383.1	possible hemolysin A	<i>Corynebacterium lipophiloflavum</i> DSM 44291	45.3	188	2.0E-55

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309789597	ZP_07684178.1	hemolysin A	Oscillochloris trichoides DG6	47.1	189	3.0E-55
332670186	YP_004453194.1	hemolysin A	Cellulomonas fimi ATCC 484	46.9	190	3.0E-55
291459862	ZP_06599252.1	hemolysin A	Oribacterium sp. oral taxon 078 str. F0262	44.3	188	3.0E-55
311742403	ZP_07716212.1	cytotoxin/hemolysin	Aeromicrobium marinum DSM 15272	47.3	188	4.0E-55
323358624	YP_004225020.1	rRNA methylase	Microbacterium testaceum StLB037	46.4	187	4.0E-55
239986743	ZP_04707407.1	putative rRNA methylase	Streptomyces roseosporus NRRL 11379	46.2	187	6.0E-55
357392624	YP_004907465.1	unnamed protein product	Kitasatospora setae KM-6054	46.6	187	7.0E-55
158320638	YP_001513145.1	hemolysin A	Alkaliphilus oremlandii OhILAs	45.3	187	7.0E-55
291443684	ZP_06583074.1	rRNA methylase	Streptomyces roseosporus NRRL 15998	46.5	187	8.0E-55
182439506	YP_001827225.1	rRNA methylase	Streptomyces griseus subsp. griseus NBRC 13350	46.5	187	1.0E-54
359737710	EHK86633.1	hemolysin A	Saccharomonospora azurea SZMC 14600	46.3	188	1.0E-54
302528025	ZP_07280367.1	ribosomal RNA large subunit methyltransferase J	Streptomyces sp. AA4	45.9	187	1.0E-54
377557512	ZP_09787155.1	hemolysin	Gordonia otitidis NBRC 100426	44.7	186	1.0E-54
152967093	YP_001362877.1	hemolysin A	Kineococcus radiotolerans SRS30216	47.9	187	2.0E-54
326780170	ZP_08239435.1	hemolysin A	Streptomyces griseus XylebKG-1	46.5	186	2.0E-54
359423443	ZP_09214578.1	hemolysin	Gordonia amarae NBRC 15530	45.2	186	2.0E-54
340359833	ZP_08682305.1	cytotoxin/hemolysin	Actinomyces sp. oral taxon 448 str. F0400	45.9	186	3.0E-54
359776863	ZP_09280166.1	hypothetical protein ARGLB_051_01780	Arthrobacter globiformis NBRC 12137	44.4	186	3.0E-54
386775382	ZP_10097760.1	putative rRNA methylase	Brachybacterium paraconglomeratum LC44	43.9	186	3.0E-54
300781249	ZP_07091103.1	cytotoxin/hemolysin	Corynebacterium genitalium ATCC	46.5	185	4.0E-54

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			33030			
262091784	ACY25372.1	hemolysin A	uncultured actinobacterium	45.8	185	4.0E-54
331697604	YP_004333843.1	hemolysin A	Pseudonocardia dioxanivorans CB1190	47.1	185	5.0E-54
29833038	NP_827672.1	rRNA methylase	Streptomyces avermitilis MA-4680	45.7	185	5.0E-54

¹The open reading frame of g0647 was determined as described {Kosuge et al., 2006, DNA Res, 13, 245-54} and BLASTed against the NCBI data base (nr, 20120604).

²E-value of 0 to 1E-100 equals high homology; E-value of 1E-100 to 1E-50 equals medium homology; E-value of 1E-50 to 1E-30 equals low homology; E-values of >1E-30 equals no homology.

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APPENDIX 11

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Final Version 1

SR-BTT FINAL REPORT 0609FR04
Summarized version of SR-BTT 0609FR02

**IN VITRO EVALUATION OF PROBIOTIC BACTERIAL STRAINS ON
BIOCOMPATIBILITY WITH HUMAN ERYTHROCYTES**

Applicant: Numico Research B.V.
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Project: 0609/02

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INTRODUCTION

Numico Research (Biomedical Research Department, section Gut Biology Microbiology) is investigating the use of probiotic bacterial strains in food products. As part of the evaluation of selected strains, the biocompatibility with human blood of these strains has to be determined.

To determine the compatibility of probiotic bacterial strains with human blood it has to be investigated if these strains or their released products change the characteristics of human blood cells, specifically platelets (see report 0609FR03) and erythrocytes (see this report). With respect to platelets, specific attention should be given to possible aggregation induction and with respect to erythrocytes, specific attention should be given to possible alpha- or beta-hemolytic activity.

SR-BTT has several standard tests available to evaluate effects of additions (like bacteria or their supernatant) on the *in vitro* quality of human blood cells. In first instance known positive and negative strains with respect to platelet and erythrocyte interaction have been used in these standard tests at SR-BTT. Subsequently the compatibility of probiotic bacterial strains with human blood cells was tested with a limited set of tests.

This report gives a summary of these tests with human erythrocytes, without the details as described in the original SR-BTT Final Report 0609FR02.

BACKGROUND INFORMATION ON TESTS PERFORMED

Physical changes

Changes in pH can affect the *in vitro* quality of erythrocytes. Because bacteria can rapidly grow, very often resulting in acidification of the medium, it has to be checked if the pH during incubation with test materials (in this study bacteria or spent media) is not too much changing compared to the control. Otherwise, possible changes can be attributed to interaction with the test materials, whereas in fact they are a result of the acidification. Because acidification can be caused by formation of lactate (produced from glucose), it can be helpful to analyse also the amount of glucose and lactate.

Hematological parameters:

These are standard hematological parameters (and standard quality parameters for products to be transfused) needed to characterize the final products. Changes in hematocrit, hemoglobin concentration and number of erythrocytes indicate dilution of the RCC during treatment (and/or significant cell loss), changes in MCV (mean cellular volume) indicate significant shape and/or volume changes induced by the treatment. The number of platelets is an indication for desintegration of erythrocytes (vesicles from desintegrated erythrocytes are counted as platelets). In leukodepleted RCC, platelets are very low or even below detection limit (i.e. $2 \times 10^6/\text{ml}$) and usually remain low, because there is no production of platelets in RCC. However, if RCC are damaged, for example by adding membrane damaging agents, or in case of severe bacterial contamination, the number of 'platelets' is increased during storage. In spiking experiments it is shown that in the Coulter Counter used, erythrocyte vesicles were picked up as platelets (although also bacteria can be counted in the platelet channel). During normal storage or upon maltreatment, vesicles may be formed after budding from the plasma membrane and they offer also a procoagulant surface to clotting factors. There is no guideline or limit set to the number of vesicles that may be present in RCC, but to a limited degree they are already formed during cold storage of RCC, especially if not leukodepleted. Preferably, the number of vesicles should not exceed the numbers normally observed in non-leukodepleted

RCC after 35 days of storage.

Hemolysis

Release of hemoglobin by lysis (hemolysis, also called β -hemolysis) is the most important RCC quality parameter, which must stay below the threshold of 0,8% according to national and international guidelines. This limit is not only to ensure that sufficient red cells remain in the product, but is also meant as safeguard against thrombogenic effects in the recipient, since broken or leaky red cells provide a procoagulant surface for clotting factors. The amount of hemoglobin is measured by multi-wavelength spectrophotometry, with correction for the presence of plasma and is an indication for integrity of the erythrocyte.

metHb formation (α -hemolysis)

Oxidation of hemoglobin by external conditions, resulting in the formation of met-hemoglobin (metHb) is called α -hemolysis. The formation of metHb as a read-out for α -hemolytic activity was measured with the OSM-3 (Radiometer, Copenhagen). The OSM-3 calculates the percentage metHb based on spectral data (with correction for other hemoglobin forms).

Phosphatidylserine

Exposure of phosphatidylserine (PS) on the outer plasma membrane is the only other mechanism (besides vesicle) by means of which red cells can have a thrombogenic effect in the recipient. PS exposure on RCC is correlating with prothrombinase activity and therefore, measurement of PS exposure with AnnexinV, is a good alternative for measurement of partial thromboplastin time (indicator for coagulation activation). Damage to the cells may result in exposure of PS, which in healthy red cells is kept on the inside of the plasma membrane to prevent this. PS exposure can readily be measured by analyzing the binding of fluorescent-labeled Annexin V (Annexin V-FITC) in a flow cytometer. PS exposure on red cells is a trigger for removal from the circulation and in normal red cells the exposure of PS is therefore minimal.

Morphology

Morphology is measured because possible changes in aggregability and deformability are always accompanied by shape changes of the cells. The erythrocyte morphology is a microscopic observation of the fixed erythrocytes (with 1% glutar-aldehyde; only judged for selected samples), with a differentiation between normal (tube-shaped) and thornapple-like erythrocytes (expressed as percentage echinocytes), which is low for fresh erythrocytes, increases during storage and/or maltreatment and can be restored upon warming. During the microscopic evaluation the samples are also screened on the presence of aggregates.

RESPONSIBILITIES

The project was carried out in the Blood Transfusion Technology Laboratory (BTT; main location U209) of the Department of Blood Cell Research (A.J.Verhoeven, Ph.D., manager) at Sanquin Research, a division of the Sanquin Blood Supply Foundation (abbreviated as SR-BTT).

As study director and SR-BTT contact was acting Dr. D. de Korte (head SR-BTT).

The study was subject to auditing by C.W.N.Gouwerok (QA responsible person SR-BTT).

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As contact for the applicant was acting Dr. R.D. Wind

MATERIALS

Numico Research supplied the necessary bacterial strains (as liquid stationary phase cultures and resuspended in PBS (pH adjusted to 6.7 with HCl)), spent culture medium (medium from stationary phase cultures, obtained after centrifugation and adjusted with HCl to pH 6.7) and plain culture medium (control, pH adjusted to 6.7), ready for use in the experiments at SR-BTT.

All other materials are standard materials used by the Sanquin Blood Centre Region North West for blood collection and by SR-BTT for blood processing and testing.

EXPERIMENTAL DESIGN

RCC units (used as test solution)

Standard RCC units in additive solution SAG-M (Saline-adenine-glucose-mannitol) were prepared at BTT-SR from whole blood units collected by Sanquin Blood Bank North West, distributed over several days. The RCC were leukodepleted by filtration and stored at 2 - 6 °C in a blood refrigerator at BTT-SR before the experiments with bacteria. Maximal storage time till use was 4 days (till 7 days RCC are defined as fresh).

RESULTS

In all tests performed with the positive controls for alpha- and beta-hemolysis as a result of incubations with bacteria, the results at 37°C could be reproduced with incubations at room temperature (RT). Because in earlier studies with incubations at 37°C some bacteria showed strong effects on pH and glucose availability at the end of the incubations, it was decided to continue with incubations at RT.

Based on the initial test results, strain E169 was chosen as positive control for α -hemolysis and strain S41 was chosen as positive control for β -hemolysis.

For the tests with probiotic bacterial strains, 6 bacterial strains were tested: one α -hemolytic strain (positive control for met-hemoglobin formation; E169), one β -hemolytic strain (positive control for cell lysis; S41) and four probiotic strains (L598, L208, B419 and B602)(see table 1).

For all strains both the bacterial suspension and the spent culture medium were tested. As a control the erythrocytes were incubated with culture medium alone and the bacteria were also diluted in PBS instead of RCC.

The effect of bacteria or the culture medium of bacteria after centrifugation (spent medium) was tested on the *in vitro* quality of RCC after incubation at 22°C during 1-4 hour. In case of culture medium the medium was brought on pH 6.7 before transport to Sanquin and sterile filtered over a 0.22 μ filter.

For the incubations with bacteria a RCC with a hematocrit of 30% was used, with 10% spiking using a bacterial suspension of 10x10⁹/ml (final ratio of about 1 bacterium per 3 erythrocytes). For the experiments with spent media a RCC with a hematocrit of 60% was diluted to 30% with spent medium (pH 6.7).

Table 1. Bacterial strains used and identification of samples

Strain (Numico code)	Specification	Bacteria spike	Spent medium
<i>E. faecium</i> (E169)	Alpha-hemolytic	1	8
<i>S. aureus</i> (S41)	Beta-hemolytic	2	9
<i>L. rhamnosus</i> (L208)	Probiotic strain	3	10
<i>L. paracasei</i> (L598)	Probiotic strain	4	11
<i>B. longum</i> (B419)	Probiotic strain	5	12
<i>B. breve</i> (B602)	Probiotic strain	6	13
Culture medium	Control	7	14

The *in vitro* quality of erythrocytes after incubation with bacteria was judged by the following parameters:

1. physical changes (measured after 1 and 4 h incubation at 22°C)
2. hematocrit, amount of hemoglobin, number of erythrocytes, mean cellular volume (MCV), number of platelets (as indication for vesiculation of erythrocytes). Measured after 1 and 4 h incubation at 22°C.
3. free hemoglobin in the resuspension medium (β -hemolysis), measured after 1 and 4 h incubation at 22°C.
4. analysis of met-hemoglobin (metHb) formation (α -hemolysis) with OSM-3 device after 1 and 4 h incubation at 22°C.
5. Binding of Annexin-V (phosphatidyl-serine (PS) exposure), measured after 1 h incubation at 22°C.
6. Erythrocyte morphology, formation of echinocytes was checked.

Results per test for erythrocytes:

1. pH and glucose/lactate

The results for the pH and glucose (from blood gas analyzer) are shown in Table 2. Bacteria are actively metabolizing, resulting in a decreased pH and glucose concentration. After 60 min only limited effect was found on pH and glucose content for incubations with bacteria, but after 210 min at 22°C a decrease for these parameters was found, especially for the first 4 bacteria strains tested. The lactate concentrations increased in parallel with the glucose decrease (not shown). For L208 the bacteria metabolized all the available glucose, resulting in a significant pH decrease (due to the lactate production).

Table 2: pH and glucose data for the various RCC incubations at RT

Strain	pH				Glucose (mM)			
	Added bacteria		Spent medium		Added bacteria		Spent medium	
	60 min	240 min	60 min	240 min	60 min	240 min	60 min	240 min
E169	6.55	6.15	6.70	6.69	7.6	2.6	10.5	10.3
S41	6.57	6.37	6.73	6.71	7.9	5.7	10.7	10.2
L208	6.57	6.03	6.70	6.67	7.2	< 0.5	10.7	10.4
L598	6.61	6.17	6.75	6.73	7.9	3.1	10.4	10.1
B419	6.76	6.74	6.69	6.65	9.8	9.4	10.9	10.7
B602	6.78	6.71	6.71	6.68	9.7	9.2	11.0	10.9

Incubations at RT; pH measured at 37°C in a Rapidlab 860 blood gas analyzer; glucose in mM as measured in a Rapidlab 860 blood gas analyzer; gray background: control (fresh medium)

The erythrocyte suspensions diluted with spent medium showed only minimal changes in pH, glucose and lactate. For the 4 h incubations with L208 and L598 it cannot be excluded that the changes in pH and/or lack of glucose will be responsible for effects on *in vitro* quality of the erythrocytes.

2. Hematological parameters

The Advia 2120 hematology counter produces more than 440 different parameters, from which the most important ones were analyzed and shown in the full report 0609FR02 (not shown in this summarized report). Some of the differences between RCC without addition (control; only diluted) and with bacteria spiked RCC were also found when bacteria were simply diluted in PBS instead of spiked into RCC. From the results it was concluded that the Advia results were not easily interpretable and that the Advia analyses could not be used as a simple tool to evaluate the biocompatibility of probiotic bacteria with human erythrocytes.

3. β -Hemolysis

The results with respect to β -hemolysis are shown in table 3. Only for S41 (positive control) the incubations with RCC showed some hemolysis, although the absolute values were relatively low. The highest degree of β -hemolysis found was 0.17, which is very close to the normal value (normal value is < 0.1 % for fresh concentrates and about 0.4 % at the end of storage for 35 days). For the spent media, only the medium derived from *S.aureus* induced β -hemolysis, with the highest value after 60 min incubation. After 240 min a minimal increase in the degree of β -hemolysis was found for the incubation with spent medium from B602. With the spent media there might be some interference with the spectrophotometric analysis method, because RCC diluted with medium showed a slightly negative value for free hemoglobin.

Table 3. Degree of hemolysis for RCC incubations at RT

Strain	Bacteria		Spent medium	
	60 min	240 min	60 min	240 min
<i>E. faecium</i> (E169)	0.05	0.07	0.08	0.05
<i>S. aureus</i> (S41) *	0.10	0.17	0.32	0.19
<i>L. rhamnosus</i> (L208)	0.06	0.11	0.05	0.08
<i>L. paracasei</i> (L598)	0.07	0.10	0.03	0.05
<i>B. longum</i> (B419)	0.07	0.08	0.05	0.05
<i>B. breve</i> (B602)	0.09	0.12	0.04	0.10
	0.05	0.05	0.00	0.02

Data expressed as % hemolysis; * β -hemolytic strain; gray background: control (fresh medium)

4. methHb formation (α -hemolysis)

The formation of methHb as a read-out for α -hemolytic activity was measured with the OSM-3 (Radiometer, Copenhagen) and is shown in Table 4. The OSM-3 calculates the percentage methHb based on spectral data (with correction for other hemoglobin forms). Significant methHb formation (normal values << 2 %) was detected in the samples incubated with E169 (positive control) and L208.

With spent medium no effects on methHb formation were detected, although nearly all incubations with spent medium resulted in a slightly higher percentage of methHb compared to the fresh medium control.

Table 4. Data on metHb formation at RT

Strain	Bacteria added		Spent medium added	
	60 min	240 min	60 min	240 min
<i>E. faecium</i> (E169) *	4.1	12.8	1.1	1.0
<i>S. aureus</i> (S41)	0.6	0.5	1.0	1.0
<i>L. rhamnosus</i> (L208)	18.1	29.8	1.2	1.2
<i>L. paracasei</i> (L598)	0.9	1.2	1.3	1.4
<i>B. longum</i> (B419)	0.7	0.8	1.2	1.4
<i>B. breve</i> (B602)	1.0	0.9	1.4	1.3

Data expressed as % metHb; * α -hemolytic strain; gray background: control (fresh medium)

5. Phosphatidylserine exposure

The percentage of erythrocytes expressing phosphatidylserine (PS, measured with AnnexinV) was analyzed. Samples A1 – A7 in table 5 are the 60 min incubations with bacterial suspensions. Only for E169 a minimal increase of the percentage erythrocytes positive for AnnexinV was found.

Samples B1 – B7 in table 5 are the 60 min incubations with spent medium. The spent medium of E169 induced a slight increase, but the incubation with medium derived from a stationary phase culture of S41 induced a high percentage of positive cells. With S41 a considerable part of the cells was already lysed upon start of the incubation. However, also during the incubation the erythrocytes were still lysing, because upon reanalyzing the sample for S41 on the FACS the percentage positive cells was decreased to 16.4 % and a longer time was needed to analyze 5000 events.

Table 5. Percentage erythrocytes positive for AnnexinV

Sample	% positive cells	Bacterial strain	Sample	% positive cells
A1	1.3	<i>E. faecium</i> (E169)	B1	0.9
A2	0.2	<i>S. aureus</i> (S41)	B2	45.0
A3	0.3	<i>L. rhamnosus</i> (L208)	B3	0.2
A4	0.3	<i>L. paracasei</i> (L598)	B4	0.2
A5	0.5	<i>B. longum</i> (B419)	B5	0.1
A6	0.2	<i>B. breve</i> (B602)	B6	0.2

Data expressed as % cells positive for Annexin-FITC binding; gray background: control (fresh medium)

6. Erythrocyte morphology

For the evaluation of erythrocyte morphology fixed samples were judged under the microscope and about 200 cells were scored, either as discocytes (normal shaped erythrocytes) or echinocytes (more swollen to discoid and presence of extrusions). Table 6 shows the percentage echinocytes in the various samples after 240 min incubation (after 60 min similar results were obtained but not shown).

The 240 min incubation with bacteria suspensions (samples A8 – A14) showed a minimal effect on the morphology of erythrocytes. For some bacteria a decrease in percentage echinocytes was detected (improved morphology; table 6).

After 240 min incubation with spent medium (samples B8 – B14) the morphology of the erythrocytes was improved for all tested media (except S41 medium) compared to the control incubation with fresh medium (table 6). In the presence of S41 the erythrocytes

showed a shrank appearance (smaller cells) and the scoring of discocytes and echinocytes could not reliable be performed (this phenomenon was found previously in study 0509FR01).

No explanation could be found for the improved morphology of the erythrocytes during incubation with bacteria or spent medium from bacteria cultures, but it was concluded that the bacteria or media had no negative effects on the erythrocytes morphology (except for the spent medium of S41).

Table 6. Percentage echinocytes in the RCC after 240 min incubation at RT

Sample	% echinocytes	Bacterial strain	Sample	% echinocytes
A8	12	<i>E. faecium</i> (E169)	B8	18
A9	39	<i>S. aureus</i> (S41)	B9	-
A10	6	<i>L. rhamnosus</i> (L208)	B10	12
A11	1	<i>L. paracasei</i> (L598)	B11	6
A12	8	<i>B. longum</i> (B419)	B12	12
A13	30	<i>B. breve</i> (B602)	B13	11

Data expressed as % echinocytes; gray background: control (fresh medium). For B9 the erythrocytes appeared shrank and the sample could not be scored reliable.

CONCLUSIONS

Biocompatibility with erythrocytes

Incubations with bacteria

- With incubations at 22°C changes in pH and glucose were much less compared to the incubations at 37°C. For some bacteria the changes after 240 min were to such an extent that the low pH and/or lack of glucose could have a negative effect on the *in vitro* quality of erythrocytes.
- The α -hemolytic strain *E. faecium* (E169) was found positive in the metHb formation tests, whereas the β -hemolytic strain *S. aureus* (S41) was found positive in the hemolysis test with bacteria or spent medium.
- The *L. rhamnosus* (L208) probiotic strains showed α -hemolytic activity for the bacteria, but not for the spent medium. Because the effects for L208 were seen already for the 60 min incubation, these negative effects cannot be related to the low pH and/or lack of glucose after 240 min.
- *B. longum* (B419) was found positive with respect to AnnexinV binding, although the reaction was minimal.

Incubations with spent media

- From the spent media, only the spent medium from the β -hemolytic strain (*S. aureus*) was positive in the hemolysis and AnnexinV binding tests. Also this medium showed an effect on the morphology of erythrocytes (smaller, shrank cells).
- Other changes in erythrocyte *in vitro* quality parameters are not judged as being of significance, because the changes are very limited and/or only visible after prolonged incubation.

Based on the results of this study it is concluded that from the tested probiotic strains *L. rhamnosus* (L208) had some negative effects on the erythrocyte *in vitro* quality, whereas

the strains *L. paracasei* (L598), *B. longum* (B419) and *B. breve* (B602) showed no significant negative effects.

RECORD MAINTENANCE

Research protocols, investigation plans and a copy of the Report (all including modifications), raw laboratory data and all correspondence between SR-BTT and the applicant will be maintained within a file at SR-BTT. These records will be retained for a period of ten years following submission of the authorized Report to the applicant.

APPROVALS

The underlying Final Report was evaluated by the head of BTT on the suitability of the experimental design and techniques used for these studies. The studies were further evaluated by putting the results in perspective of the general knowledge on the in vitro evaluation of blood components.

I accept responsibility for the conduct of of this study.

.....
Dr. D. de Korte
Head Blood Transfusion Technology Laboratory
Department of Blood Cell Research

.....
Date

Statement of Manager Blood Cell Research
I agree with the conclusions of this SR-BTT Report

.....
Dr. A. J. Verhoeven
Manager Department of Blood Cell Research

.....
Date

Statement of Applicant
We accept the SR-BTT report 0609FR04.001:

.....
Dr. R.D. Wind
Numico Research

.....
Date

QUALITY ASSURANCE

Procedures and facilities are subject to regular internal auditing by the department of QA of the divisions "Diagnostic Services" and "Research" of Sanquin.

The Dutch Council for accreditation operating as accreditor for test laboratories declares that SR-BTT complies with the accreditation criteria for laboratories as described in NEN-

EN-ISO/IEC 17025:2005 (certificate with accreditation number L 266 was granted for the first time on September 30, 1998).

CCKL, the Dutch Accreditation Board for Medical Laboratories declares that SR-BTT complies with the accreditation criteria for laboratories as described in the CCKL guidelines which contain the criteria from ISO 15189:2003, ISO 17025:2005 and ISO 9000:2000 (accreditation number 81 was granted for the first time on October 17, 2002).

QUALITY ASSURANCE STATEMENT

The study described in this Final Report was audited by me and was found to be performed according to the research protocol and investigation plans.

.....
C.W.N. Gouwerok
QA responsible person SR-BTT

.....
Date

APPENDIX 12

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Final Version 1

SR-BTT FINAL REPORT 0609FR03
Summarized version of SR-BTT 0609FR01

**IN VITRO EVALUATION OF PROBIOTIC BACTERIAL STRAINS ON
BIOCOMPATIBILITY WITH HUMAN BLOOD PLATELETS**

Applicant: Numico Research B.V.
Biomedical Research Department, section Gut Biology Microbiology
Bosrandweg 20
PO Box 7005
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The Netherlands

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Sanquin Research
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Project: 0609/01

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INTRODUCTION

Numico Research (Biomedical Research Department, section Gut Biology Microbiology) is investigating the use of probiotic bacterial strains in food products. As part of the evaluation of selected strains, the biocompatibility with human blood of these strains has to be determined.

To determine the compatibility of probiotic bacterial strains with human blood it has to be investigated if these strains or their released products change the characteristics of human blood cells, specifically platelets (this report) and erythrocytes (see report 0609FR04). With respect to platelets, specific attention should be given to possible aggregation induction and with respect to erythrocytes, specific attention should be given to possible alpha- or beta-hemolytic activity.

SR-BTT has several standard tests available to evaluate effects of additions (like bacteria or their supernatant) on the *in vitro* quality of human blood cells. In first instance known positive and negative strains with respect to platelet and erythrocyte interaction have been used in these standard tests at SR-BTT. Subsequently the compatibility of probiotic bacterial strains with human blood cells was tested with a limited set of tests.

This report gives a summary of these tests with human platelets, without the details as described in the original SR-BTT Final Report 0609FR01.

BACKGROUND INFORMATION ON TESTS PERFORMED

Aggregation tests

Platelets can be induced to aggregation by several agonists like ADP, collagen and thrombin. This can be measured in an aggregometer, by changes in the transmission of the platelet suspension. Without aggregation the transmission is low (disturbed by the discoid platelets), whereas after aggregation the transmission increases, due to the clumping. However, interaction with test materials (in this study bacteria and spent media from bacterial cultures) can also result in spontaneous aggregation, which is described for several bacterial strains (for example for Staphylococcus strains). Because this process is relatively slow compared to agonist induced aggregation, the aggregation curve has to be followed for about 25 min, in contrast to the 5 min it takes for a normal aggregation curve after addition of an agonist.

Hematological parameters (platelet count):

These are standard hematological parameters (and standard quality parameters for products to be transfused) needed to characterize the final products. Changes in MPV (mean platelet volume) indicate significant shape and/or volume changes induced by the treatment. The number of platelets is an indication for disintegration and/or clumping of platelets. In leukodepleted PC, leukocytes are very low or even below detection limit (i.e. $2 \times 10^6/\text{ml}$) and usually remain low, because there is no production of leukocytes in RCC. However, if PC are activated, for example by adding bacteria, the number of 'leukocytes' can increase during storage, because platelet clumps are picked up as 'leukocytes'.

Physical changes

Changes in pH can affect the *in vitro* quality of erythrocytes. Because bacteria can rapidly grow, very often resulting in acidification of the medium, it has to be checked if the pH during incubation with test materials (in this study bacteria or spent media) is not too much changing compared to the control. Otherwise, possible changes can be attributed to

interaction with the test materials, whereas in fact they are a result of the acidification. Because acidification can be caused by formation of lactate (produced from glucose), it can be helpful to analyse also the amount of glucose and lactate.

Phosphatidylserine

Exposure of phosphatidylserine (PS) on the outer plasma membrane is the only other mechanism (besides vesicle) by means of which red cells can have a thrombogenic effect in the recipient. PS exposure on RCC is correlating with prothrombinase activity and therefore, measurement of PS exposure with AnnexinV, is a good alternative for measurement of partial thromboplastin time (indicator for coagulation activation). Damage to the cells may result in exposure of PS, which in healthy red cells is kept on the inside of the plasma membrane to prevent this. PS exposure can readily be measured by analyzing the binding of fluorescent-labeled Annexin V (Annexin V-FITC) in a flow cytometer. PS exposure on red cells is a trigger for removal from the circulation and in normal red cells the exposure of PS is therefore minimal.

RESPONSIBILITIES

The project was carried out in the Blood Transfusion Technology Laboratory (BTT; main location U209) of the Department of Blood Cell Research (A.J.Verhoeven, Ph.D., manager) at Sanquin Research, a division of the Sanquin Blood Supply Foundation (abbreviated as SR-BTT).

As study director and SR-BTT contact was acting Dr. D. de Korte (head SR-BTT).

The study was subject to auditing by C.W.N.Gouwerok (QA responsible person SR-BTT)

As contact for the applicant was acting Dr. R.D. Wind

MATERIALS

Numico Research supplied the necessary bacterial strains (as liquid stationary phase cultures and resuspended in PBS (pH adjusted to 6.7 with HCl)), spent culture medium (medium from stationary phase cultures, obtained after centrifugation and adjusted with HCl to pH 6.7) and plain culture medium (control, pH adjusted to 6.7), ready for use in the experiments at SR-BTT.

All other materials are standard materials used by the Sanquin Blood Centre Region North West for blood collection and by SR-BTT for blood processing and testing.

EXPERIMENTAL DESIGN

PC units (used as test solution)

Standard platelet concentrates (PC) in plasma (derived from single buffy coats) were prepared at BTT-SR from whole blood units collected by Sanquin Blood Bank North West, distributed over several days. The PC had a low leukocyte content (due to the preparation method) and were stored at 22 ± 1 °C in a platelet incubator (shaking) at BTT-SR until the experiments with bacteria, which were performed on the same day as the PC was prepared.

RESULTS

The effect of bacteria was tested on the *in vitro* quality of platelets after incubation at 22°C (to prevent strong effects on pH), although the aggregation tests were performed at 37°C (standard conditions).

Bacterial suspensions were incubated with platelets during 60 min or during 180 min. Single donor platelet concentrates were only used after being tested upon positively in initial aggregation testing with ADP as stimulus and with strains S2 and S41 as stimulus (due to donor variation not all platelet concentrates will be positive in these tests).

Table 1 shows the bacterial strains tested and the scheme for the incubations. As a control the platelets were incubated with fresh culture medium alone. The platelet concentrates were diluted with plasma (from the corresponding unit) to a concentration of about 250×10^6 /ml. For the various tests, 10 % v/v of a bacterial suspension at a concentration of 2.5×10^9 /ml was added.

Table 1. Bacterial strains used and tests performed

Strain (numicode)	PC 1	PC2	PC3	PC4
<i>S. aureus</i> (S2)	X	X	X	X
<i>S. aureus</i> (S41)	X	X	X	X
<i>L. paracasei</i> (L598)	X	X	X	X
<i>L. rhamnosus</i> (L208)	X	X	X	X
<i>L. rhamnosus</i> (L208C) (faecal isolate)			X	X
<i>B. breve</i> (B602)	X	X	X	X
<i>B. longum</i> (B419)	X	X	X	X
Fresh culture medium	X	X	X	X

S.: *Staphylococcus*; L.: *Lactobacillus*; B.: *Bifidobacterium*. PC1 and PC2 were tested during a first set of experiments, PC3 and PC4 were tested during a second set of experiments.

The *in vitro* quality of platelets after incubation with bacteria were judged by the following parameters:

1. induction of aggregation (measured after 10 min preincubation at 37°C), with visual inspection on the presence of clumps.
2. physical changes: platelet counts (hematological parameters) and blood gases (measured after 1 and 3h incubation at 22°C), including visual inspection on the presence of clumps.
3. changes in exposure of phosphatidylserine (PS; measured with AnnexinV-FITC binding after 60 min incubation at 22°C).

Results per test for platelets

1. Induction of aggregation

The aggregation response was measured in an aggregometer (at 37°C, after 10 min preincubation, under stirring at 900 rpm). The platelet concentrates used showed a normal aggregation response upon addition of ADP (40 µM) and showed aggregation after addition of *S.aureus* strains S2 or S41 (see Appendix II for some representative curves). Most of the strains tested did not show aggregation (L208, L208C B602, B419), but with L598 for PC1 some aggregation was seen, starting very late compared to the aggregation induced by S2 and S41 (table 2). For PC2 with L598 after 25 min a minimal increase

started, just at the moment that (according to the protocol) ADP was added, which resulted in a normal aggregation. For PC3 and PC4, L598 showed some response, with a quick initial increase in transmission, but no further reaction during the 25 min waiting period and a normal aggregation response upon subsequent addition of ADP (see appendix I for all curves with L598). This reaction was different compared to what was found during the first series of experiments. During this series for PC1 a delayed, but real aggregation response was found, whereas for PC2 a slight increase in transmission was found after 25 min, probably not reflecting real aggregation (because still normal aggregation was detected upon ADP addition). In addition, spent media from all bacterial strains were tested, but for none of the spent media aggregation was induced (not shown).

Table 2. Aggregation results

		Additions							
	Parameter	ADP	S2	S41	L59 8	L208	B60 2	B41 9	L208 C
PC 1	Tmax.	4	3	21	25	None	None	None	Nt
	T up	0,5	1,2	9	20,5	None	None	None	Nt
	max-min	43	45	50	26	1	-3	1	Nt
PC 2	Tmax.	4	5	21	>25	None	None	None	Nt
	T up	0	1	8	24	None	None	None	Nt
	max-min	35	37	43	-4	1	-2	-0,5	Nt
PC 3	Tmax.	4,5	5	3	25	none	none	none	None
	T up	0,25	0	0,5	0,5	none	none	none	None
	max-min	23	46	15	5	0	0	0	0
	clumps	large	large	large	None	none	none	none	None
PC 4	Tmax.	2	5	4	25	none	none	none	None
	T up	0,25	0	0,5	0,5	none	none	none	None
	max-min	26	42	12	3	-1	0	0	0
	clumps	large	large	large	None	none	none	none	None

Tmax: time to reach maximal aggregation

Tup: time between addition and start of aggregation (increase in curve)

Max-min: distance between minimum and maximum transmission (%)

None: no aggregation; Nt: not tested

2. Physical parameters

The number of platelets was counted in a Advia 2120 (Bayer) hematological counter. In addition to the platelet count, this counter gives a lot of additional information. However, due to interference of bacteria and platelet clumps with the platelet counts, no useful additional information could be extracted from these data. Therefore, these data are not discussed in this summary, but are included in the underlying full report 0609FR01. All incubations with bacteria were checked on the presence of visual aggregates. Platelets incubated with either L598 and L208 (also L208C) contained small clumps after 60 min incubation, which were much larger after 180 min for L598, but not for the L208 strains. The Advia 2120 was able to discriminate between WBC and platelet aggregates, because the number of WBC always remains below $0.05 \times 10^6/\text{ml}$ (the platelet concentrates used did not contain leukocytes). The RCC count was $< 0.05 \times 10^9/\text{ml}$ for all samples.

Bacteria are actively metabolizing, resulting in a decreased pH and glucose concentration and an increase in the lactate concentration. For all incubations the supernatant was tested on pH and glucose and lactate content at the end of incubation. The pH after 60 min varied from 7.2 – 7.6 (start 7.04 – 7.20), with minimal changes in glucose and lactate content. The slight increase in pH on itself will have no negative effect on the *in vitro* quality of platelets. After 180 min incubation the pH values varied from 7.06 – 7.43, still with minor changes in glucose and lactate content. These changes can not explain the effects on *in vitro* quality of platelets.

3. PS-exposure

The percentage of platelets expressing phosphatidylserine (PS, measured with AnnexinV) was analyzed. Normal resting platelets have a very low percentage positive cells for this marker, because PS is actively kept inside (asymmetric distribution in the membrane). For the incubations with *S.aureus* strains and platelets after 60 min at RT, the percentage of platelets positive for AnnexinV was increased (normal value is below 3%) for all donors tested (except PC3 with S41). For the two lactobacillus strains there was a slight increase with most donors tested, whereas for the bifidobacterium strains only a minimal effect of the incubation was found (table 3). Incubation with only culture medium (fresh, adjusted to pH 6.4) showed a minimal increase for AnnexinV binding compared to the control with PBS.

Table 3. PS-exposure on platelets induced by bacteria

	Annexin-V % pos				Interpretation
	PC 1	PC 2	PC3	PC4	
<i>S. aureus</i> (S2)	22.4	17.7	23,4	10,4	Positive
<i>S. aureus</i> (S41)	25.6	17.4	3	6	Positive to slightly positive
<i>L. paracasei</i> (L598)	8.5	6.1	9,7	8,3	Positive
<i>L. rhamnosus</i> (L208)	5.4	6.1	2,1	1,7	Slightly positive to negative
<i>L. rhamnosus</i> (L208C) (faecal isolate)			2,4	2,3	Negative
<i>B. breve</i> (B602)	2.0	2.5	4.5	2.7	Negative
<i>B. longum</i> (B419)	2.2	2.7	2.0	1.6	Negative
Medium	4.0	3.0	3.3	2.0	Slightly positive to negative

% positive cells for Annexin-V-FITC binding, medium is fresh culture medium. Gray background: control (PBS), nt: not tested

CONCLUSIONS

Biocompatibility with platelets

Incubations with bacteria

- The positive controls S2 and S41 were positive in all the tests used: aggregation, hematological counter, visual clumping and degree of platelets positive for AnnexinV binding.
- With L598 all tests used showed some positive response. For PS-exposure all different PC were positive, but for aggregation only two out of 4 tested PC

- were positive, whereas the effect with respect to induction of visual clumping or counting differed in time scale.
- With L208 (two different isolates), the results were very variable, with some reactivity in the AnnexinV binding assay (only in the first series of experiments) and some effect on platelet count (associated with visual clumping).
 - In most of the tests the B419 and B602 strains were negative, but with B602 in the second series of experiments some reactions with platelets were found, although variable per donor and not very strong.

Based on the results of this study it is concluded that from the tested probiotic strains *L. paracasei* (L598) had some negative effects on the platelet *in vitro* quality, whereas the strains *L. rhamnosus* (L208), *B. longum* (B419) and *B. breve* (B602) showed no significant negative effects.

RECORD MAINTENANCE

Research protocols, investigation plans and a copy of the Report (all including modifications), raw laboratory data and all correspondence between SR-BTT and the applicant will be maintained within a file at SR-BTT. These records will be retained for a period of ten years following submission of the authorized Report to the applicant.

APPROVALS

The underlying Final Report was evaluated by the head of BTT on the suitability of the experimental design and techniques used for these studies. The studies were further evaluated by putting the results in perspective of the general knowledge on the *in vitro* evaluation of blood components.

I accept responsibility for the conduct of of this study.

.....
Dr. D. de Korte
Head Blood Transfusion Technology Laboratory
Department of Blood Cell Research

.....
Date

Statement of Manager Blood Cell Research
I agree with the conclusions of this SR-BTT Report

.....
Dr. A. J. Verhoeven
Manager Department of Blood Cell Research

.....
Date

Statement of Applicant
We accept the SR-BTT report 0609FR03.001:

.....
Dr. R.D. Wind
Numico Research

.....
Date

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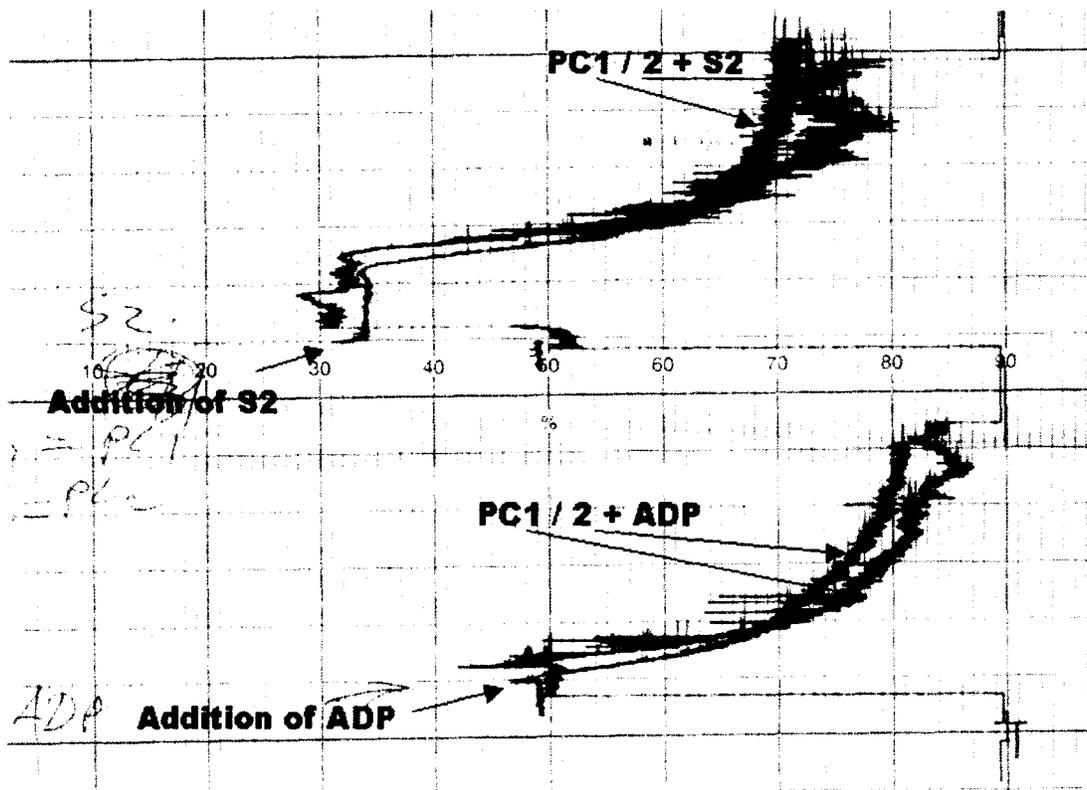
QUALITY ASSURANCE STATEMENT

The study described in this Final Report was audited by me and was found to be performed according to the research protocol and investigation plans.

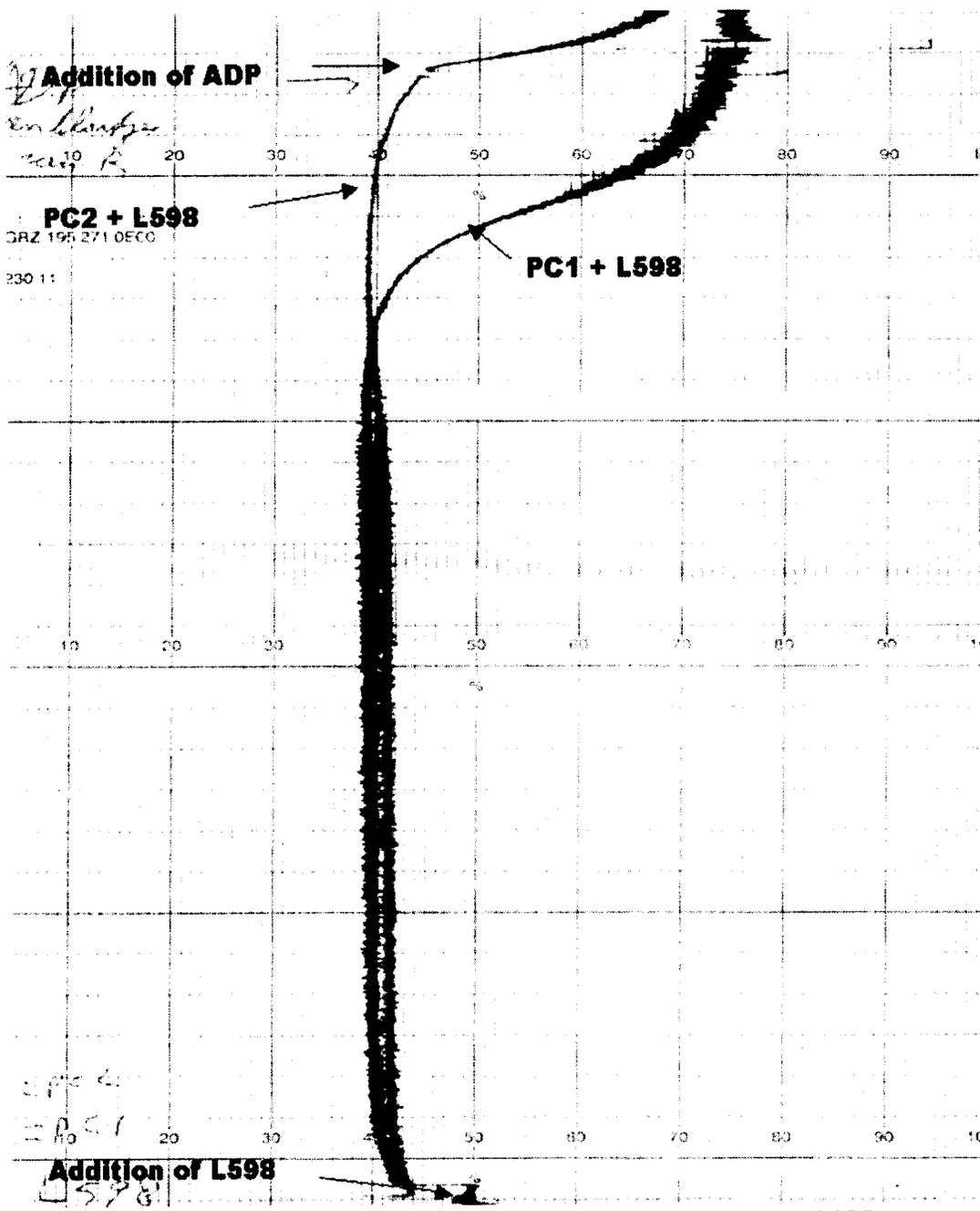
.....
C.W.N. Gouwerok
QA responsible person SR-BTT

.....
Date

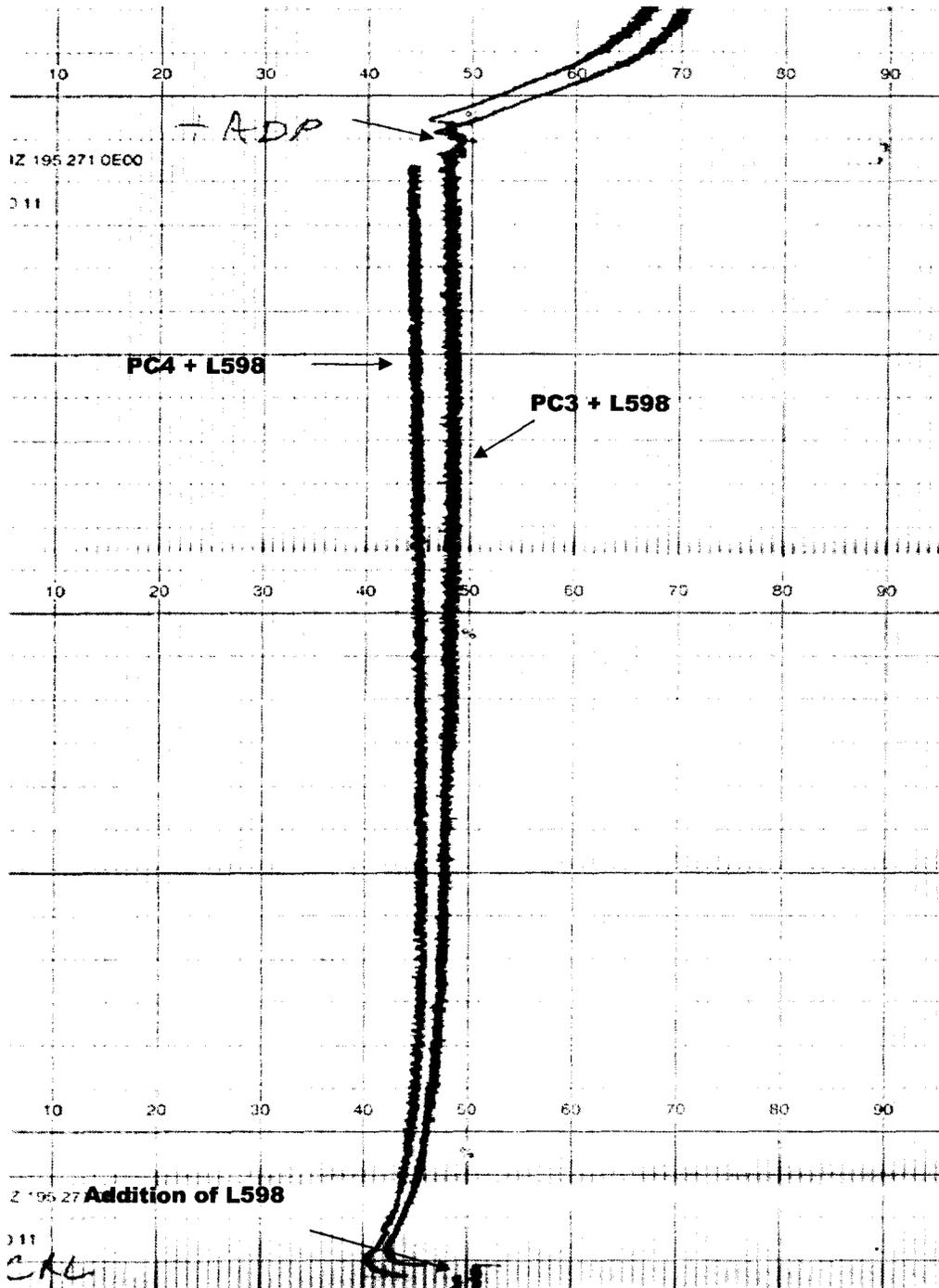
Appendix I: some representative aggregation curves



Curves for addition of ADP or S2 to PC1 and PC2 with complete aggregation

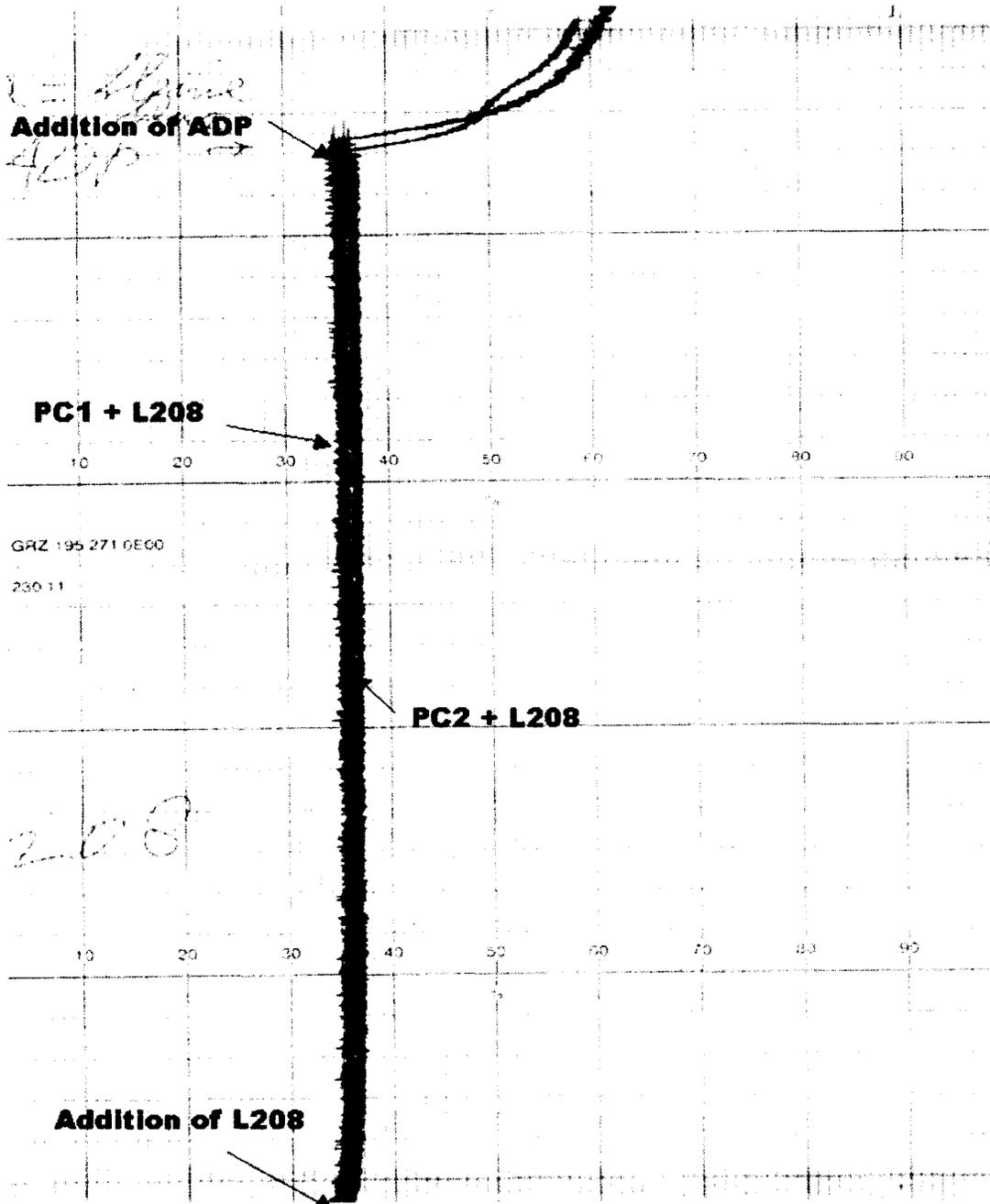


Curves after addition of L598 to PC1 and PC2, with after 25 min addition of ADP to check if (further) aggregation can be induced



Curves after addition of L598 to PC3 and PC4, with after 25 min addition of ADP to check if (further) aggregation can be induced

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Curves after addition of L208 to PC1 and PC2, with after 25 min addition of ADP to check if normal aggregation can be induced. Similar curves were found with B419 and B602.

**GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR
Bifidobacterium breve M-16V IN SELECTED CONVENTIONAL AND MEDICAL
FOODS**

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of food and food ingredients, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of the use of *Bifidobacterium breve* M-16V (“*B. breve* M-16V”) in selected food products for the general population and medical foods containing hydrolyzed proteins and/or amino acid mixtures for children. These data and information are summarized in the GRAS determination document, Generally Recognized As Safe (GRAS) Determination for *Bifidobacterium breve* M-16V in Selected Conventional and Medical Foods, produced by Spherix Consulting, Inc., for Morinaga Milk Industry Co., Ltd. This GRAS determination was made based on scientific procedures for GRAS determinations described under 21 CFR §170.30(b).

Based upon our review of the information and data available, we find that the intake of *B. breve* M-16V from the intended uses specified has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that *B. breve* M-16V is safe, and GRAS, under the intended conditions of use, the safety of the intake of *B. breve* M-16V has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of *B. breve* M-16V as an ingredient in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. Bifidobacteria are naturally occurring gut microbiota in infants and adults. *B. breve* has been detected in the stools of infants and adults.
2. Bifidobacteria have been consumed in fermented foods for decades and current commercial strains include *Bifidobacterium animalis* (*B. animalis*) ssp. *lactis* strain Bf-6, *Bifidobacterium lactis* (*B. lactis*) Bb-12, *B. lactis* DR10 (HN019), *Bifidobacterium longum* (*B. longum*) BB536, *B. breve* Yakult, *B. breve* SBT-2928, and *B. breve* C50. *B. breve* M-16V was first commercially available in Japan in 1976.
3. In the United States, various *Bifidobacterium* species have been determined to be GRAS for use in conventional foods and infant formulas, including: *B. animalis* ssp. *lactis* Bf-6 for use in selected foods (GRN 377; 10¹¹ cfu/serving of conventional

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foods); *B. lactis* Bb-12 for use in infant formulas for four months-of-age and older (GRN 49; 10^7 - 10^8 cfu/g infant formula) and *B. longum* BB536 for use in selected foods and infant formulas (GRN 268; 10^{10} cfu/serving of conventional foods; 10^{10} cfu/g of term infant formula).

4. *Bifidobacterium breve* M-16V is a Gram-positive anaerobic bacterium. This organism was deposited with the Belgian Co-ordinated Collections of Micro-organisms (BCCM) and designated LMG 23729.
5. The original frozen culture of *B. breve* M-16V is tightly controlled to ensure purity and genetic stability of the strain.
6. Product specifications assure that *B. breve* M-16V is suitable for use in conventional and medical foods.
7. Finished products made with *B. breve* M-16V cultures reproducibly meet compositional standards and comply with limits on contaminants appropriate for food-grade ingredients.
8. *B. breve* M-16V meets the safety standards enumerated by the Food and Agriculture Organization of the United Nations/World Health Organization's (FAO/WHO) guidelines for the evaluation of microbes for probiotic use in foods. Results show that *B. breve* M-16V is not toxic or pathogenic and is therefore safe for use in foods:
 - a. The genome of *B. breve* M-16V does not contain regions with significant homology to known antibiotic resistance genes, including those found in other strains of bifidobacteria and lactobacilli.
 - b. Functional assays indicate that *B. breve* M-16V exhibits antibiotic susceptibility patterns similar to the type strain *B. breve* ATCC 15700.
 - c. *B. breve* M-16V does not contain plasmids.
 - d. *B. breve* M-16V produces predominantly L-lactic acid, while production of D-lactic acid is negligible.
 - e. *B. breve* M-16V hydrolyzes the conjugated bile acids taurocholic and glycocholic acid to the primary bile acid cholic acid and hydrolyzed glycochenodeoxycholic and taurochendeoxycholic acid to chenodeoxycholic acid.
 - f. *B. breve* M-16V does not dehydroxylate cholic acid and chenodeoxycholic acid to the secondary bile acids deoxycholic and lithocholic acid.
 - g. *B. breve* M-16V does not produce biogenic amines.
 - h. *B. breve* M-16V does not produce ammonia.

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- i. *B. breve* M-16V does not have azoreductase or nitroreductase activity.
 - j. *B. breve* M-16V does not have hemolytic activity.
 - k. *B. breve* M-16V has no deleterious effects on platelet aggregation or viability.
 - l. *B. breve* M-16V has no mucolytic activity.
9. Toxicology studies show no evidence of *B. breve* M-16V translocating the gut epithelium, it does not induce mutations in *Salmonella typhimurium* strain TA98 and TA100 with or without metabolic activation, and does not produce test article-related toxicity in a 90-day repeated dose oral toxicity study in rats administered 2.3×10^{11} cfu *B. breve* M-16V/kg/day.
 10. Twelve studies published from 1992 – 2012 have reported the safe administration of *B. breve* M-16V at doses up to 1.5×10^{10} cfu/d for durations of up to 3 months in a total of 430 health compromised and/or premature infants. *B. breve* M-16V was well tolerated and no treatment-related adverse health effects were noted.
 11. *B. breve* M-16V strain has been administered in two studies in adults at a dose of 2×10^{10} cfu/d for 4-8 wk in patients with intermittent to mild persistent asthma or atopic dermatitis. There were no treatment-related adverse effects noted.
 12. Other, non-M-16V strains of *B. breve* have been administered to adults and children at doses up to 8×10^{11} cfu/d for durations up to 1 yr and no adverse treatment-related effects were reported.
 13. Assuming a maximum addition of 5.0×10^9 cfu *B. breve* M-16V/serving of the conventional foods listed in this document, the estimated mean and 90th percentile 2-day average intakes of *B. breve* M-16V from all categories combined in the population ages 2 years and older are 3.8×10^{10} and 6.0×10^{10} cfu/day, respectively.
 14. Under the most conservative assumptions, a maximum addition of 10^8 cfu of *B. breve* M-16V per gram of powdered medical foods containing hydrolyzed proteins and/or amino acid mixtures for children ages one to ten years old, the estimated daily intake of a one year-old and a ten year-old is 1.6×10^{10} cfu and 4.2×10^{10} cfu *B. breve* M-16V per day, respectively.

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Determination of the GRAS status of *B. breve* M-16V under the intended conditions of use has been made through the deliberations of A. Wallace Hayes, PhD, DABT; Gregor Reid, PhD, MBA, ARM, CCM, Dr HS, FCAHS; Roger Clemens, Dr PH, CNS, FACN, FIFT. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of *B. breve* M-16V and the human exposure to *B. breve* M-16V resulting from its intended use as an ingredient in selected conventional foods and medical foods containing hydrolyzed proteins and/or amino acid mixtures for children, and have concluded:

There is no evidence in the available information on B. breve M-16V that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when B. breve M-16V is used at levels that might reasonably be expected from the proposed applications. B. breve M-16V is GRAS for use in selected conventional and medical foods as proposed by Morinaga Milk Industry Co., Ltd.

Therefore, *B. breve* M-16V is safe and GRAS at the proposed levels of addition to foods. *B. breve* M-16V is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

It is our opinion that other experts qualified by training and/or experience to evaluate the safety of food and food ingredients would concur with these conclusions.

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SUBMISSION END

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