APPENDIX A

US FDA STANDARD PLATE COUNT GUIDANCE

DILUTING SAMPLES

1. Work Area
    a. Level plating bench not in direct sunlight.
    b. Sanitized immediately before start of plating.
2. Selecting Dilutions
    a. Plate two decimal dilutions per sample.
    b. Select dilutions to yield one plate with 25-250 colonies.
3. Identifying Plates
    a. Label each plate with sample identification and dilution.
    b. Arrange plates in order before preparation of dilutions.
4. Sample Agitation
    a. When appropriate, wipe top of unopened containers with sterile, ethyl alcohol-saturated cloth.
    b. Remove test portion within 3 min of sample agitation.
5. Sample Measurement, pipets
    a. Use separate sterile pipets for the initial transfers from each container.
        1. Pipets in pipet container adjusted without touching the pipets.
    b. Pipet tip not dragged over exposed exterior of pipets in container.
    c. Pipet not dragged across lip or neck of sample container.
    d. Pipet not inserted more than 2.5 cm (1") below sample surface (foam avoided if possible).
    e. Draw test portion above pipet graduation mark and remove pipet from liquid.

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1. Pipet aid used, mouth pipetting not permitted.

f. Adjust test volume to mark with lower side of pipet in contact with inside of sample container (above the sample surface).

g. Drainage complete, excess liquid not adhering to pipet.

h. Release test portion to petri dish (tip in contact with plate, 45°angle) or dilution blank (with lower side of pipet in contact with neck of dilution blank, or dry area above buffer where appropriate) with column drain of 2-4 sec.

i. Blow out last drop of undiluted sample from pipet using pipet aid

1. Blow out away from main part of sample in plate, do not make bubbles.

j. Pipets discarded into disinfectant, or if disposable into biohazard bags or containers to be sterilized.

6. Dilution Agitation

a. Optionally, use approved mechanical shaker for 15 sec.

b. Remove test portion within 3 min of dilution agitation.

7. Dilution Measurement, pipets

a. Use separate sterile pipets for the initial transfers from each container.

1. Pipets in pipet container adjusted without touching the pipets.

b. Pipet tip not dragged over exposed exterior of pipets in container.

c. Pipet not dragged across lip or neck of dilution blank.

d. Pipet not inserted more than 2.5 cm (1") below dilution surface.

e. Draw dilution portion above pipet graduation mark and remove pipet from liquid.

1. Pipet aid used, mouth pipetting not permitted.

f. Adjust dilution volume to mark with lower side of pipet in contact with inside of dilution blank neck.

g. Drainage complete, excess liquid not adhering to pipet.

h. Gently lift cover of petri dish just high enough to insert pipet.

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i. Hold pipet at 45°angle to dish with tip touching dish (or dilution blank neck).

j. Release dilution portion to dish (or dilution blank) with tip in contact with the bottom of the dish (or dilution blank neck, or dry area above buffer where appropriate) with column drain of 2-4 sec.

k. Touch pipet tip once against dry spot on dish bottom (or dilution blank neck).

  l. When measuring 0.1 mL, do not re-touch dry area.

m. Pipets discarded into disinfectant, or if disposable into biohazard bags or containers to be sterilized.

PLATING

8. Plating

  a. Melt agar quickly in boiling water, flowing steam not under pressure, or microwave oven (use extreme caution when microwaving).

  b. Avoid prolonged exposure to high temperatures during and after melting, establish lab protocol.

  c. Do not melt more than will be used within 3 hours.

  d. Do not melt agar more than once.

  e. Promptly cool melted agar to 45±1°C.

    1. Record temperature with other control information.

  f. Temperature control used for each test medium type.

    1. Contains medium identical to type being used.

    2. In container identical to that being used.

    3. Undergoes same heat treatment and cooling as test medium.

  g. Select number of samples in any series so that all will be plated within 20 min after diluting first sample.

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h. After depositing test portions, promptly pour 10-12 mL medium into each plate of series, or 15-20 mL for > 1 mL portion/plate or where agar weight loss is a problem that can not be corrected by other actions (documentation must be kept to indicate that this is a routine practice).

i. Lift cover of petri dish just high enough to pour medium.

j. As each plate is poured thoroughly and evenly mix medium and test portion in petri dish.

   1. Multiple plates may be poured and mixed, however, plates may not be stacked prior to mixing.

k. Allow to solidify within 10 min on level surface.

<u>CONTROLS</u>

9. Controls

   a. Check sterility of dilution blanks, medium, petri dishes, and pipets used for each group of samples.

<u>COUNTING COLONIES</u>

10. Counting Aids

   a. Count colonies with aid of magnification under uniform and properly controlled artificial illumination with a hand tally.

11. Recording Standard Plate Count

   a. After incubating plates, promptly count all colonies on selected plates.
   b. Where impossible to count at once, store plates at 0-4.4°C for not longer than 24 h (avoid as a routine practice).
   c. Record dilutions used and number of colonies on each plate counted.
   d. Record results of sterility and control tests.
   e. When possible, select spreader free plates with 25-250 colonies and count all colonies.

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1. Use higher magnification if necessary to distinguish colonies from foreign matter.
2. Examine edge of petri plates for colonies.

f. If consecutive plates yield 25-250 colonies, count all colonies on plate(s) from both dilutions.

g. Count chains from separate sources as separate colonies.

h. If there is no 25-250 colony plate, use plate having nearest to 25 or 250 colonies.

i. If plates from all dilutions exceed 250 colonies, estimate counts as follows

1. Count colonies in portions representative of distribution and estimate total.
2. Where there are < 10 colonies/sq cm, count colonies in 12 squares, selecting 6 consecutive squares horizontally across the plate and six consecutive squares at right angles.
3. When there are 10 or more colonies/sq cm, count 4 representative squares.
4. Multiply average number colonies/sq cm by area of plate in sq cm.

j. If plates yield < 25 colonies each, record actual number in lowest dilution.

k. If all plates from a sample show no colonies, record count as 0.

12. Personal Errors

a. Avoid inaccurate counting due to carelessness, fatigue, or impaired vision.

b. Discover cause and correct if unable to duplicate your own counts on the same plate.

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REPORTS

20. Computing and Reporting Counts.

a. Multiply number of colonies (or estimated number if necessary) by the reciprocal of the dilution.

b. If consecutive dilutions yield 25-250 colonies, compute count using formula below (see current SMEDP).

$$N = \Sigma C / [(1 \times n_1) + (0.1 \times n_2)]\, d$$

Where, N = number of colonies per milliliter or gram

ΣC = sum of all colonies on all plates counted

n_1 = number of plates in lower dilution counted

n_2 = number of plates in next highest dilution counted

d = dilution from which the first counts were obtained

Example: 1:100 = 244 colonies 1:1,000 = 28 colonies

$$N = (244 + 28) / [(1 \times 1) + (0.1 \times 1)]0.01$$

$$= 272 / [1.1]0.01$$

$$= 272 / 0.011$$

= 24,727 [25,000 (reported)]

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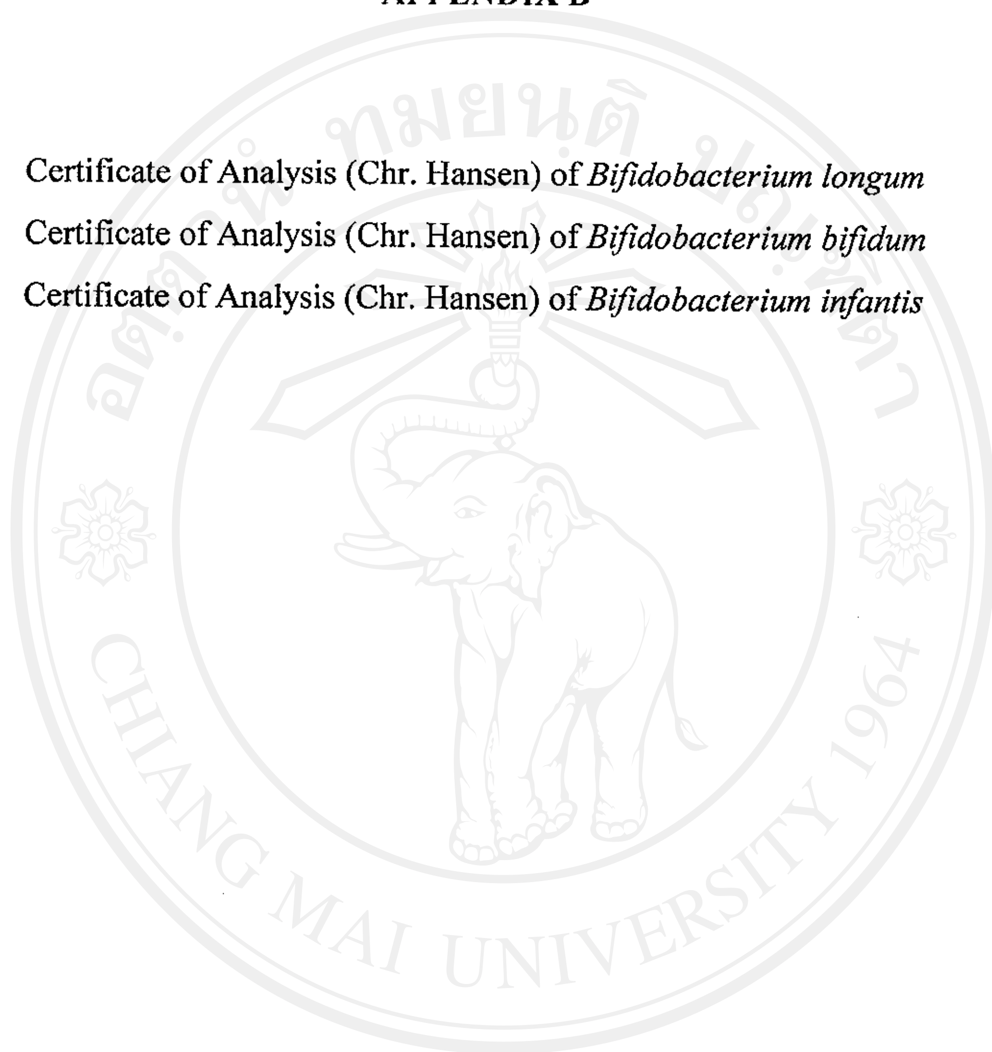
APPENDIX B



- Certificate of Analysis (Chr. Hansen) of *Bifidobacterium longum*
- Certificate of Analysis (Chr. Hansen) of *Bifidobacterium bifidum*
- Certificate of Analysis (Chr. Hansen) of *Bifidobacterium infantis*

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Chr. Hansen, Inc.
16300 West Lincoln Avenue
New Berlin, WI 53151
Telephone: 414-607-5700
Fax: 262-814-2133

Certificate of Analysis

Manufactured Date: 10/22/2002

Item Name:. B.Longum 10 Billion 5 kg.
Item Number: 602595
Lot Number: 2430718

Test Name / Method	Specification	Test Results
Coliform	<10	<1
E.Coli	Negative	Negative
S.Aureus	Negative	Negative
Salmonella	Negative/25gm.	Negative
Yeast & mold	<100/gm	<100/gm
Listeria	Negative / 25 gm.	Negative
Cell Count - label guarantee	> 10 billion	>10 billion

Comments:

Authorized By: Karen Sylvester

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Chr. Hansen, Inc.
16300 West Lincoln Avenue
New Berlin, WI 53151
Telephone: 414-607-5700
Fax: 262-814-2133

Certificate of Analysis

Manufactured Date: 01/06/2003

Item Name:. Bifidobacterium BB-12 10 billion 5 Kg.
Item Number: 602594
Lot Number: 2444482

Test Name / Method	Specification	Test Results
Coliform	<10	<1
E.Coli	Negative	Negative
S.Aureus	Negative	Negative
Salmonella	Negative/25gm.	Negative
Yeast & mold	<100/gm	<100/gm
Listeria	Negative / 25 gm.	Negative
Cell Count - label guarantee	> 10 billion	>10 billion

Comments:

Authorized By: Karen Sylvester

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CHR HANSEN

Chr. Hansen, Inc.
16300 West Lincoln Avenue
New Berlin, WI 53151
Telephone: 414-607-5700
Fax: 262-814-2133

Certificate of Analysis

Manufactured Date: 01/23/2003

Item Name:. B.Infantis 10 billion 5 Kg.
Item Number: 602596
Lot Number: 2441169

Test Name / Method	Specification	Test Results
Coliform	<10	<1
E.Coli	Negative	Negative
S.Aureus	Negative	Negative
Salmonella	Negative/25gm.	Negative
Yeast & mold	<10	<10
Listeria	Negative / 25 gm.	Negative
Cell Count - label guarantee	> 10 billion	>10 billion

Comments:

Authorized By: Karen Sylvester

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	1992 Bachelor of Science degree in Agro-industry, King Mongkut's Institute of Technology, Lardkrabang
Experiences	- Production supervisor, the Prachuap Fruit Canning Company, Prachuapkeereekhun, Thailand (10 months)
	- Research and development officer, the Laemthong Food Industries Company, Nakornpathom, Thailand (2 years)

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