AN ABSTRACT OF THE THESIS OF

RONSHAN CHENG for the degree of MASTER OF SCIENCE in FOOD SCIENCE AND TECHNOLOGY presented on DECEMBER 1, 1989

Title: Growth, Physiological Characteristics and Plasmid Profiles of Bifidobacterium Species

Abstract approved: _____
William Sandine

The fecal flora of healthy bottle or breast-fed infants was examined for the presence of Bifidobacterium. Identification was based on the presence of fructose-6-phosphate phosphoketolase, which is found only in these bacteria. No bifidobacteria were recovered from bottle-fed infants. However, bifidobacteria were readily isolated from 15 day to 3 month old breast-fed infants. Further characterization revealed B. breve and B. longum were the dominant species in feces of breast-fed infants, but atypical strains also were found.

A whey-based medium (7% sweet whey, 0.05% cysteine and 0.3% yeast extract, WCY-0.3) was developed to grow Bifidobacterium species without use of anaerobic incubation conditions. Freshly pasteurized WCY-0.3 was inoculated with 0.2% (10^6 to 10^7 CFU/ml) of the following active cultures of bifidobacteria: B. bifidum 15696, B. breve 15700, B. longum 15707, B. breve 15698, B. longum L10, B. longum L12, and B. longum 3j. Following incubation for 12 hours, most strains reached cell densities of 10^9 to 5 x 10^9 CFU/ml, except

B. bifidum 15696 and B. longum 3j. Addition of Oxyrase to the WCY (WC with any level of yeast extract) at 0.03 unit/ml (WCYO) reduced the lag phase of all strains, allowing maximum populations to be reached more quickly. A higher population density (2 to 7 times) could be achieved in the WCOY-0.3 medium with strains 15696, 15700, 15707, and L10 by incorporating 1.9% sodium glycerophosphate or trimagnesium phosphate with incubation for 12 hours at 37°C. Also, viability of these strains was retained throughout a 24-hour incubation period, in contrast to rapid death of cells grown without the neutralizing agents. Inoculation of WCY-0.3 or WCOY-0.3 medium with frozen concentrates (10^7 to 10^8 CFU/ml) of bifidobacteria allowed equal growth of all species, except B. bifidum 15696, which grew much better in WCOY-0.3 than in WCY-0.3. Survival stability of whey-based medium-grown bifidobacteria when resuspended in pasteurized skim milk and refrigerated at 4°C was strain dependent and enhanced by the presence of 0.05% cysteine; generally ATCC strains were more stable than strains freshly isolated from baby feces. In this regard, B. breve 15700, B. longum 15707, and B. breve 15698 did not lose viability in 11% skim milk with 0.05% cysteine within 10 days of storage. Stability of whey-based medium-grown bifidobacteria in WCY with 15% glycerol during six months storage at -40°C was strain dependent. Bifidobacterium bifidum 15696, B. breve 15700, B. longum 15707, B. breve 15698, and B. longum L12 did not lose viability; however B. bifidum L6 lost about 50% viability, while B. longum L10, B. breve T10, and B. breve T2 lost about one log population density.

The plasmid profiles of 35 strains of bifidobacteria from human sources were examined. Only one strain, B. breve 15698, harbored a 5.8Kb plasmid. A curing process using UV-light treatment to remove the plasmid was carried out but characterictics of the cured strain were identical to those of the parent strain, indicating the plasmid is cryptic.

Growth, Physiological Characteristics and Plasmid Profiles of Bifidobacterium Species

by

Ronshan Cheng

A thesis

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Completed November 10, 1989

Commencement June 1991

APPROVED:

Professor of Microbiology in charge of major

Chairman of Food Science and Technology Department

Dean of Graduate School

Date thesis is presented DECEMBER 1, 1989

ACKNOWLEDGEMENTS

I would like to express appreciation to my adviser, Dr. W. E. Sandine, for his guidance and assistance throughout this investigation. I am also grateful to him for the use of his laboratory and facilities.

I wish to thank The Western Dairy Foods Research Center, Logan, Utah for generously providing the grant which supported this project and made it possible.

Finally, I thank all the graduate students in the department of Microbiology Dairy Lab who helped me at one time or another throughout this research project.

Table of Contents

List of Figures

List of Tables

GROWTH, PHYSIOLOGICAL CHARACTERISTICS AND PLASMID PROFILES OF BIFIDOBACTERIUM SPECIES

CHAPTER 1

GROWTH CHARACTERISTICS OF BIFIDOBACTERIUM IN WHEY BASED-MEDIUM

ABSTRACT

A whey-based medium (7% sweet whey, 0.05% cysteine and 0.3% yeast extract, WCY-0.3) was found to be satisfactory for growth of a variety of Bifidobacterium species without use of anaerobic incubation conditions. Freshly pasteurized (90°C , 45 min) and cooled (37°C) WCY-0.3 was inoculated with 0.2% (10^6 to 10^7 CFU/ml) of the following active cultures of bifidobacteria: B. bifidum 15696, B. breve 15700, B. longum 15707, B. breve 15698, B. longum L10, B. longum L12, and B. longum 3j. Following incubation for 12 hours, most strains reached maximum cell densities of 10^9 to 5 x 10^9 CFU/ml, except B. bifidum 15696 and B. longum 3j. Addition of oxyrase to the WCY(WC with any level of yeast extract) at 0.03 unit/ml (WCYO) reduced the lag phase of all strains, allowing maximum populations to be reached more quickly. Boiled oxyrase (1%) was found to be a growth promoter for B. longum 15707, but not for the other strains. Two to seven times population density could be achieved in the WCOY-0.3 medium with strains 15696, 15700, 15707, and L10 by incorporating 1.9% sodium glycerophosphate or trimagnesium phosphate with incubation for 12 hours at 37°C. Also, viability of these strains was retained throughout a 24-hour incubation period, in contrast to rapid death of cells grown without the neutralizing agents. Inoculation of WCY-0.3 or WCOY-0.3 medium with frozen concentrates (10^7 to 10^8 CFU/ml) of B. bifidum 15696, B. breve 15700, B. longum 15707, B. breve 15698, B. longum L10, or B. longum T10 allowed equal growth of all species, except B. bifidum

15696, which grew much better in WCOY-0.3 than in WCY-0.3. Bifidobacterium longum L10 inoculated at 5 x 10^6 CFU/ml had a lag phase in WCY-0.3, but grew vigorously in WCOY-0.3. Survival stability of WCYO-grown bifidobacteria when resuspended in pasteurized skim milk and refrigerated at 4°C was strain dependent and enhanced by the presence of 0.05% cysteine; generally ATCC strains were more stable than strains freshly isolated from baby feces. In this regard, B. breve 15700, B. longum 15707, and B. breve 15698 did not lose viability in 11% skim milk with 0.05% cysteine within 10 days of storage. Stability of WCYO-grown bifidobacteria in WCY-0.3 with 15% glycerol during six months storage at -40°C was strain dependent. Bifidobacterium bifidum 15696, B. breve 15700, B. longum 15707, B. breve 15698, and B. longum L12 did not lose viability; however B. bifidum L6 lost about 50% viability, while B. longum L10, B. breve T10, and B. breve T2 lost about one log population density.

INTRODUCTION

Bifidobacterium is a genus of bacteria originally classified as lactobacilli. Since 1957, they have been classified in the genus Bifidobacterium on the basis of carbohydrate fermentation, physiological characteristics, and DNA-homology pattern (20,21). Bifidobacterium are the dominant organisms in stools of breast-fed infants and also are found in the human vagina, mouth, and in the alimentary tracts of various animals (21). They are gram-positive rods but often stain irregularly, especially in older cultures. Freshly isolated strains may be branched, bifurcated Y and V, or spatulate shape, but may become straight or curved rods of various widths and lengths and may break to resemble branching when cultured in the laboratory (20). Several factors have been reported to induce pleomorphism, such as sodium chloride and chlorides of other univalent cations. Other reports suggest that branching in bifidobacteria was principally due to an inability to form cross walls when grown in a medium deficient in calcium ions (21). The exact mechanism of branching in bifidobacteria is still unknown.

Bifidobacteria are thought to play a significant health role in the intestines of infants. Lower morbidity and mortality are seen in breast-fed infants than in bottle-fed infants, which has led many people to study the health role and ecology of bifidobacteria (28). The most frequent pathogens causing neonatal infections are coliform organisms. Human milk promotes the growth of bifidobacteria, and this suppresses the growth of coliform and other pathogenic bacteria

and confers resistance to gastro-intestinal infections in neonates; thus, breast-feeding is considered beneficial to infant health (8,24).

Most bifidobacteria are able to hydrolyze conjugated bile acids, such as sodium taurcholate and glycocholate (9). Therefore, it has been suggested that bifidobacteria play an important role in transformation of bile acids in the intestine. They may accentuate the capacity of bile salts to inhibit susceptible bacteria (9,21).

Freeze-dried B. bifidum (10^7 cells/day) have been added to the diet of formula-fed infants and used as supplementary treatment in some enteric infections (21). Bifidobacterium longum also has been reported to develop a " barrier effect" in a germ free rat model, against pathogenic E. coli (8). Ammonia produced in the intestine is the main contributor to hepatic coma (21); bifidus milk containing viable B. bifidum has been reported to produce an improvement in liver cirrhosis patients by causing decreases in ammonia, free phenols and fecal pH along with an increase in feces bifidobacterial content. Also, bifidobacteria have been reported to be important for vitamin synthesis in humans (7) .

Although bifidobacteria have these potential nutritional and therapeutic effects, they are not popular in the U.S. as either dietary adjuncts or therapeutic agents. They are used to make cultured milk in Europe and sold in Japan as food supplements in the form of freeze-dried powders. These bacteria are nutritionally fastidious, heterogeneous and anaerobic or microaerophilic in the presence of carbon dioxide. Some strains sensitive to oxygen require a low oxidation-reduction potential for growth and fermentation. However, the need for anaerobiosis is reported to be different for different

strains (21,25). Thus, they are difficult to grow. Media reported to support vigorous growth are expensive and/or not acceptable for addition to foods. Growth in milk with the addition of modified MRS broth or malt extract added has been reported (5,17). Whey is an abundant and relatively inexpensive by-product from cheese processing and can cause pollution problems. Whey represents not only a serious environmental problem but frequently an unnecessary waste of valuable food materials (27). Based on this knowledge, a whey-based medium was investigated in an effort to culture Bifidobacterium in a simple and inexpensive manner.

MATERIALS AND METHODS

CULTURES

Bifidobacterium bifidum 15696, B.breve 15700, B. longum 15707, and B. breve 15698 used in this study were obtained from American Type Culture Collection, Rockville, MD; B. longum L10, B. longum L12, B. breve T11, B. breve T2, and B. bifidum L6 were isolated from feces of 15 days old breast-fed infants (for characteristics of Bifidobacterium spp. isolated from babies stools, please see Chapter 2 for further information). Bifidobacterium longum 3j was isolated from a commercial preparation of "bifidus powder" (Calpis Food Industry Co., LTD Japan). All strains were maintained by weekly transfer in Reinforced Clostridial Medium (RCM) incubated anaerobically at 37°C for 12 hours. Before being inoculated into the testing medium, the microorganisms were initially grown in the RCM medium for 12 hours; the activated strains provided a 0.2% inoculum for the testing medium. Unless otherwise noted, activated organisms were used for all experiments.

REAGENTS AND EQUIPMENT

Materials used and source: Sweet whey (Tillamook County Creamery Association, Tillamook, Oregon); yeast extract (Busch Industrial Products Corporation, St. Louis, Missouri); oxyrase enzyme system (Oxyrase Inc., Ashland, Ohio); sodium glycerophosphate (Ruger Chemical Co., Inc, Irvington, N.J.); glycerol (EM Science);

potassium hydroxide (Mallinckrodt); malt extract (Difco); evaporated milk (Carnation Brand). Cysteine, sodium phosphate dibasic, sodium phosphate monobasic, and sodium chloride were purchasd from Sigma Chemical Co. Items of equipment were from these sources: Multi-stir (Lab-line Multi-Magnetic); incubator shaker (New Brunswick Scientific Co., Inc); water bath (American Scientific Products); cryovials (West Coast Scientific, Inc); stomacher (TekMar Company, Cincinnatic, Ohio); pH meter (Corning model 125).

MEDIA PREPARATION

Testing media were mixed well and adjusted to pH 7 with KOH before pasteurization. Aliquots (50 ml) were delivered into 50 ml flasks and pasteurized at 90°C for 45 min in a water bath. Pasteurization and cooling to 37°C were done immediately before inoculation to expel the oxygen and to create a reduced condition. Inoculum (0.2%) of activated strains (10^6 to 10^7 CFU/ml) and oxyrase were added. Media were incubated at 37°C. Samples were withdrawn at 0, 6, 12, and 24-hour intervals and promptly analyzed for viable cells and pH. Unless otherwise noted, this media preparation method was used throughout all experiments.

EXPERIMENT #1: DEVELOPMENT OF WHEY-BASED MEDIUM FOR BIFIDOBACTERIUM

Sweet whey (7%) and 0.05% cysteine were combined with different amounts of yeast extract (0, 0.1, 0.3,and 0.5%) and prepared according to the media preparation method. After pasteurization, 0.2% activated strains and different levels of oxyrase (0, 0.03,and 0.15 unit/ml) were added. A total of 12 (4 x 3) combinations of media were made at each time. Each flask was screw-capped and incubated at 37°C aerobically for 24 hr and 5 ml samples were withdrawn at 6, 12, and 24 hour intervals and analyzed for viable count and pH. During the incubation period, flasks did not receive additional agitation, except when samples were taken from flasks. Strains used in this experiment were B. bifidum 15696, B. breve 15700, B. longum 15707, B. longum L10, B. longum L12, B. breve 15698, and B. longum 3j.

EXPERIMENT #2: EFFECTS OF SODIUM GLYCEROPHOSPHATE AND CYSTEINE UNDER SHAKING OR NON-SHAKING CONDITIONS

Media containing 7% sweet whey, 0.3% yeast extract and with or without 1.9% disodium glycerophosphate or 0.05% cysteine were prepared according to the media preparation method. A combination of 4 (2 x 2) different media were made; after pasteurization, 0.2% activated strains and 0.15 unit/ml oxyrase were incorporated. Flasks

were loosely capped and incubated at 37°C with or without shaking (100 rpm). Samples were withdrawn at 6, 12, and 24 hour intervals and analyzed for viable cells and pH. Strains used in this experiment were B. bifidum 15696, B. breve 15700, and B. longum 15707.

EXPERIMENT #3: COMPARSION OF SODIUM GLYCEROPHOSPHATE AND TRIMAGNESIUM PHOSPHATE

Media consisting of 7% sweet whey, 0.3% yeast extract, and 0.05% cysteine with 1.9% sodium glycerophosphate (SG) or 1.9% trimagnesium phosphate (TP) were prepared according to the media preparation method. After pasteurization, 0.2% activated strains and 0.03 unit/ml oxyrase were added and the flasks tightly screw-capped. Two groups were incubated at 37°C, one without any agitation and the other with slow speed agitation (20 rpm, in Lab-line Multi-Magnestir). Samples were withdrawn and analyzed for viable count and pH. Strains used in this experiment were B. bifidum 15696, B. breve 15700, B. longum 15707, and B. longum L10.

EXPERIMENT #4: GROWTH FACTOR FROM OXYRASE

Sweet whey (7%) plus 0.05% cysteine used as a control basal medium was made according to the media preparation method. Oxyrase (0.3 unit/ml boiled at 100°C for 10 min) was added to the basal medium to serve as the experimental group. Aliquots (15 ml) of both media were delivered into 30 ml sterile test tubes, and each received a 0.2% inoculum. Both control and experimental tubes

were equally distributed into 3 gas-pak jars (BBL) and incubated under anaerobic conditions. Each jar was removed at 6, 12, and 24-hour intervals to analyze the viable count and pH. Strains used in this study were B. bifidum15696, B. breve 15700, B. longum 15707, B. longum L10, B. longum L12, and B. breve 15698.

EXPERIMENT #5: GROWTH COMPARISON IN EVAPORATED MILK WITH MALT EXTRACT OR YEAST EXTRACT

Medium A (evaporated milk)

Sterilized evaporated milk was combined with an equal amount of sterilized water.

Medium B (evaporated milk with 10% malt extract)

Sterilized evaporated milk was combined with an equal amount of sterilized malt extract (20%) solution.

Medium C (evaporated milk with 0.3% yeast extract)

Sterilized evaporated milk was combined with an equal amount of sterilized yeast extract (0.6%) solution.

The above three media A, B, and C, were mixed well and 15 ml aliquots of each medium were delivered into 30-ml capacity sterile culture tubes; 0.2% activated inoculum was added, and then each tube was placed into a gas-pak jar for anaerobic incubation at 37°C for 48 hours. Samples were taken at 12, 24, 36, and 48-hour intervals to analyze for viable counts and pH. Strains used in this experiment were B. bifidum 15696, B. breve 15700, B. longum 15707, and B. breve 15698.

EXPERIMENT #6: GROWTH IN WHEY-BASED MEDIUM USING STRAINS PREPARED AS FROZEN CONCENTRATES

Medium containing 7% sweet whey, 0.3% yeast extract, and 0.05% cysteine was made according to the media preparation method. After pasteurization, oxyrase (0, 0.03 unit/ml) and inoculum (0.05% of frozen-state strain, which had been frozen at -40°C for 3 months), were added. Each flask was screw-capped and incubated at 37°C without any agitation for 24 hr. Samples were removed at 6, 12, and 24 hour intervals to be analyzed for viable count and pH. Strains used in this experiment were B. bifidum 15695, B. breve 15700, B. longum 15707, B. breve 15698, B. longum L10, and B. breve T10.

EXPERIMENT #7: STABILITY OF STRAINS IN REFRIGERATED MILK WITH AND WITHOUT CYSTEINE

Seven percent sweet whey, 0.3% yeast extract, and 0.05% cysteine in 500 ml amounts were used and prepared according to the media preparation method. After pasteurization 0.03 unit/ml of oxyrase and 0.2% inoculum were added, followed by incubation at 37°C for 12 hours. Then the cells were spun down at 8000 rpm for 15 min. Pellets were placed asepectically into a zip-loc bag and resuspended in 100 ml of pasteurized 11% skim milk with or without 0.05% cysteine and the bag vacuumized 3 min to remove air. Contents were stomached 1 min to mix the cells well. Aliquots (8 ml)

were distributed into sterile 10 ml screw-capped test tubes and stored at 4°C. Samples were removed at 0, 2, 4, 6, 8, 10, 15, 20, and 40 days intervals and tested for the viable count. Strains used in this study were B. bifidum 15696, B. breve 15700, B. breve 15698, B. longum 15707, B. longum L10, B. longum L12, B. bifidum L6, and B. breve T10.

EXPERIMENT #8: STABILITY OF FROZEN CONCENTRATES DURING SIX MONTHS OF STORAGE

Culture medium and cell preparation method were the same as for experiment #7. Cell pellets were resuspended in 50 ml of sterile whey-based medium which contained 7% sweet whey, 0.3% yeast extract, 0.05% cysteine and 15% glycerol. Aliquots (4 ml) were delivered into 4 ml capacity sterile cryovials and frozen at -40°C. Samples were removed at monthly intervals and analyzed for viable count. Strains used in this experiment were B. bifidum 15696, B. breve 15700, B. longum 15707, B. breve 15698, B. longum L10, B. longum L12, B. breve T2, B. breve T10, and B. bifidum L6.

VIABLE CELL COUNT

Samples were diluted in phosphate buffered saline (PBS) solution, and 1 ml aliquots from appropriate dilutions were pour-plated into RCA and incubated at 37°C for 48 hr under anaerobic conditions (GasPak, BBL). After incubation, each plate containing 25 to 250 colonies was counted.

RESULTS

DEVELOPMENT OF A WHEY-BASED MEDIUM FOR BIFIDOBACTERIA

It was first ascertained that growth could be improved by incorporating cysteine into the medium. L-cysteine apparently reduced the oxidation-reduction potential and enhanced growth of the bifidobacteria (4, 21). Therefore, 0.05% cysteine was added to the whey-based medium. Results of growth experiments are shown in Tables 1.1 to 1.7. Most strains tested would not grow in 7% whey + 0.05% cysteine (WC). Bifidobacterium breve 15698 only maintained viability (1.0×10^7 CFU/ml at 0 hr to 4.0×10^7 CFU/ml at end of incubation) throughout the 24-hr incubation period, while B. longum 3j increased 2 logs in population (from 1.8×10^6 CFU/ml to 1.8×10^8 CFU/ml) by the end of incubation. Most strains grew slowly over the incubation period and did not reach their maximum density in WC with 0.1% YE except, B. breve 15698 and B. longum 3j which reached their maximum density at 12 hours and B. breve 15700 achieved its maximum density at end of incubation. WC with 0.3% YE or 0.5% YE did not show a noticeable difference for all tested strains; most of them achieved cell densities of 10^9 CFU/ml during 12 hr incubation, while B. bifidum 15696 and B. longum 3j only reached about 5×10^8 CFU/ml. WC or WCY incorporated with any levels of oxyrase (WCO and WCYO) helped all the strains to reduce the lag phase and allowed them to reach the stationary phase more quickly. Bifidobacterium longum 15707 in the WCO did not achieve a higher

density than in WC with any amount of YE. This suggested that WCY (0.3% or 0.5% YE) was more nutritious than the WCO. For B. bifidum 15696, the WCO was more nutritious than the WCY, which could not reach maximum cell densities without the addition of oxyrase. Most of the other strains, for example B. breve 15700, B. breve 15698, B. longum L10, B. longum L12, and B. longum 3j , did not show any difference in WCO or WCY, indicating 7% sweet whey + 0.05% cysteine + 0.3% YE (WCY-0.3) under reduced conditions could satistify the nutrient requirements for most of the tested bifidobacteria.

EFFECT OF SODIUM GLYCEROPHOSPHATE AND CYSTEINE UNDER SHAKING OR NON-SHAKING CONDITIONS

Table 1.8 illustrates the extent of bifidobacterial growth in 7% whey + 0.3% YE + 0.15 unit/ml oxyrase (WOY-0.3) supplemented with an internal buffer agent and cysteine. Results indicate cysteine was required for B. bifidum 15696, since in the WOY-0.3 medium B. bifidum 15696 did not reach its maximum population density under shaking or non-shaking conditions; also, cysteine did not show any significant influence on B. breve 15700 or B. longum 15707. Incorporating 1.9% sodium glycerophosphate helped all the tested strains maintain their viability over the incubation time. By the end of incubation, strain 15696 maintained less viable cells under shaking conditions than under non-shaking conditions; shaking or non-shaking did not cause any differences in strains 15700 or 15707. Data here implied that strain 15696 was more oxygen-

sensitive than the other two strains, because even with 0.15 unit/ml of oxyrase, it still could not reach higher population densities.

COMPARISON OF DISODIUM GLYCEROPHOSPHATE AND TRIMAGNESIUM PHOSPHATE

In WCYO (7% sweet whey, 0.05% cysteine, 0.3% yeast extract, and 0.03 unit/ml oxyrase) medium, 1.9% of disodium glycero-phosphate (SG) or trimagnesium phosphate (TP) was added as an internal pH-control agent. Results are shown in Tables 1.10 to 1.11. At the initial stage (before 6 hour), TP cultures had much higher cell densities than SG cultures, but from 12 hour until the end of the incubation time, these two buffer agents did not show a noticeable difference. These agents helped all strains tested maintain their viability, and 5×10^9 to 10×10^9 CFU/ml cell densities were achieved at end of incubation. The same strains and media were incubated under 20 rpm stirring conditions and none of them could maintain their viability (Table 1.10), indicating 0.03 unit/ml of oxyrase can not efficiently remove the continuously dissolved oxygen, and all strains therefore lost their viability gradually.

GROWTH FACTOR FROM OXYRASE

Oxyrase, a commercial product consisting of E. coli membrane fractions, can remove the dissolved oxygen in the medium. To test for growth promoters, oxyrase was boiled at 100°C for 10 min then added to the WC (7% sweet whey + 0.05% cysteine) at the 1% level.

Results are shown in Table 1.13. Most strains responded the same in media with or without boiled oxyrase, except strain 15707, where a higher viable cell count was observed in medium with boiled oxyrase added. In medium without boiled oxyrase, these cells lost their viability gradually. This indicatied WC medium provided enough nutrients for the growth of B. bifidum 15696, B. breve 15700, B. longum L10, and B. longum L12, provided the medium was maintained in a reduced conditions.

GROWTH IN EVAPORATED MILK WITH MALT EXTRACT OR YEAST EXTRACT

Malt extract (ME) in skim milk has been found to be a good culture medium for bifidobacteria (17). Therefore it was investigated in this experiment. Results are shown in Table 1.12. Evaporated milk alone did not provide enough nutrients for the growth of bifidobacteria, because most tested strains lost their viability gradually during incubation; one exception was strain 15698, which achieved and maintained an optimum population throughout the whole incubation time. Evaporated milk with 10% ME allowed very slow growth for most strains but they eventually achieved 10^8 CFU/ml cell densities except for B. breve 15698 which reached a 10^9 CFU/ml population at the end of incubation. Results in evaporated milk with 0.3% YE were strain dependent. Bifidobacterium bifidum 15696 reached 10^8 CFU/ml before 12-hour and then gradually lost viability. Strain 15700 reached its maximum density of 2×10^9 CFU/ml at 24 hours then lost viability gradually. Strain 15707

maintained a population of 10^9 CFU/ml during 24 to 36 hours, then lost its viability gradually. Strain 15698 was the only one that reached its maximum density before 12 hours and kept its population around 10^9 CFU/ml until the end of incubation.

GROWTH IN WHEY-BASED MEDIUM USING CULTURE PREPARED AS FROZEN CONCENTRATE

A 0.25-ml aliquot from 10^{-1} dilution of a frozen concentrated culture was inoculated into 50 ml of WCY-0.3 with or without 0.03 unit/ml oxyrase. Growth results are shown in Table 1.14. The initial inoculum of six strains was around 10^7 to 10^8 CFU/ml except for strain L10, which was only at 5 x 10^6 CFU/ml. Strains with a high initial inoculum number, for example 15698 (1.5 x 10^8 CFU/ml), T10 (8 x 10^7 CFU/ml), and 15707 (1.2 x 10^7 CFU/ml) did not show a significant difference in media with or without oxyrase. Strain L10, in which the initial inoculum was 5 x 10^6 CFU/ml, did show slow growth in the WCY-0.3, but it increased very quickly and reached maximum densities before 12-hour of incubation in WCOY-0.3. Strain 15696 still grew much better in the presence of oxyrase than in the medium without oxyrase. Results here indicated that: (1) oxygen-sensitivity was strain dependent (for example, strain 15696 is an oxygen-sensitive strain); (2) high inoculum overcame the oxygen-damage of cell viability and allowed the maximum growth to be achieved more quickly.

STABILITY OF BIFIDOBACTERIA IN REFRIGERATED SKIM MILK AND AS FROZEN CONCENTRATES

The stability of bifidobacteria grown in whey-based medium when stored in refrigerated 11% skim milk (with or without 0.05% cysteine) and frozen concentrates (WCY-0.3, with 15% glycerol added) is shown in Table 1.15 and Table 1.16. Stability in refrigerated milk was strain dependent and enhanced by the presence of cysteine for some strains. Generally strains from A.T.C.C. were more stable than strains freshly isolated from feces. For example, strains 15700, 15707, and 15698 did not lose their activity in the first 10 days of storage in the presence of cysteine compared to loss of 60 to 80% viability without the cysteine. Strains freshly isolated from stools, for example L10, L12, and T10, lost 50 to 98% of viability in the presence of cysteine in the first 10 days of storage. Strain 15696 was more unstable than the other A.T.C.C. strains. About 70% viability was lost within the first 10 days of storage even in the presence of cysteine. Strain L6 was the most stable among the freshly isolated strains. It did not lose any viability in the cysteine-added skim milk in the first 10 days of storage, but did lose about 50% viability in the absence of cysteine.

During 6 months storage at -40 ^{o}C, strains from A.T.C.C. did not lose their viability; in contrast, strains T2, T10, and L10 lost about one log population density.

DISCUSSION

Bibidobacteria are deemed to be benefical for human beings of all ages. In breast-fed infants, bifidobacteria maintain a relatively low pH in the large intestine, and thus provide protection against intestinal infections caused by Salmonella, Shigella, and enteropathogenic E. coli. In adults with antibiotic-induced diarrhea or liver disease, the feeding of a bifidobacterial inocula corrects these problems (21, 24). Thus, there is considerable interest in optimal growth conditions for the different types of bifidobacteria in dairy products used as dietary adjuncts. Some reports have appeared regarding the growth of bifidobacteria in milk (5,17,21), and it has been recognized that two factors are important in the cultivation of bifidobacteria: an adequate culture medium and anaerobic conditions. The purpose of the work described in this chapter was to investigate whether or not the most common human bifidobacterial species can be cultured in a simple and economic way.

GROWTH CHARACTERISTICS IN WHEY-BASED MEDIUM

It is generally accepted that bifidobacteria are strictly anaerobic and do not possess catalase activity (20); however, great differences in sensitivity to oxygen were found for different bifidobacterial strains. In this regard, our results corroborate previous reports. Strain 15698 maintained population densities during the incubation periods and strain 3j grew slowly in the basal

medium (7% whey+ 0.05% cysteine-WC), whereas most of the other strains could not maintain their viability over the incubation time. This indicates that over the sampling period, too much oxygen was dissolved and the key enzyme, fructose-6-phosphate phosphoketolase (F6PPK), was inactivated by the accumulating hydrogen peroxide (20). Survibility of all strains was improved when yeast extract(YE) was incorporated into the WC. This implies that components from YE supply growth stimulants to culture the bifidobacteria. In a recent report (19) in which different kinds of potential growth promoters were surveyed, only the YE was able to improve growth in a synthetic medium. Our results indicate that 0.1% YE in WC (WCY-0.1) was not enough for most of the tested strains, because only B. breve 15698 and B. longum 3j reached their maximum densities before 12 hour incubation. WC with 0.3% or 0.5% YE (WCY-0.3 or -0.5) were much better than WCY-0.1 for most strains in maximum population achievement, and these two media did not cause a noticable difference, except with B. longum 3j, which did not show any difference in the presence of different amounts of YE. Although the nature of YE as a growth factor has not been determined, probably oxygen damage to cells was overcome by hydrogen peroxide inactivation. In this regard it was found that all the strains in the WCY medium under slow agitation lost viability quickly. Oxyrase has been reported to allow aerobic growth of anaerobes (1,2,12); it removes the dissolved oxygen and maintains the medium in an anaerobic condition. In this experiment two different levels of oxyrase (0.03 or 0.15 unit/ml) did not make a population difference for growth of the bifidobacteria. Also, WC or

WCY incorporated with oxyrase (WCO and WCYO) helped all the strains to reduce the lag phase and allowed the maximum densities to be reached before 12 hours of incubation; an exception was strain 15707, which achieved a population of 3 x 10^8 CFU/ml before 12 hours and maintained the population over the incubation period. This indicates that for strain 15707, the extra-nutrients from YE are more important than an oxygen-free condition, because in the WCY medium, it reached a much higher population than in the WCO medium. Adversely, for strain 15696, an oxygen-free enviroment (i.e., WCO) is more important than a nutrient-fortified medium (i.e., WCY), since in the WCO medium a population of 10^9 CFU/ml was reached before 12 hours compared to the WCY medium, which reached approximately 10^9 CFU/ml by the end of incubation. The other strains (for example B. breve 15700, B. breve 15698, B. longum L10, and B. longum 3j) did not show much growth difference in WCO or WCYO. So data here indicate a simple medium (7% sweet whey + 0.05% cysteine + 0.3% yeast extract medium: WCY-0.3) under reduced conditions can satistify the growth requirements for most the bifidobacteria tested in this experiment.

EFFECT OF SODIUM GLYCEROPHOSPHATE, OR CYSTEINE UNDER SHAKING OR NON-SHAKING CONDITIONS

Cysteine is a reducing agent which lowers the oxidation-reduction potential and is routinely incorporated into media to culture anaerobes (4). It has been reported that autooxidation of cysteine results in the production of hydrogen peroxide which is toxic to some anaerobes (3). In contrast, Rolfe et al. (22) found no

evidence that oxidized products of cysteine affected the survival of numerous anaerobes used in their studies. The present data show that cysteine is required for B. bifidum 15696 as a growth factor or reducing agent, since in the WCYO medium this strain quickly reached 10^9 CFU/ml (Table 1.1) before 12 hours of incubation; however under shaking or non-shaking conditions, the WOY-0.3 (0.15 unit/ml of oxyrase without 0.05% cysteine) medium did not support as a high cell density compared to the WCOY-0.3 medium. Results here indicate that cysteine did not have a synergistic effect with oxyrase. In the initial medium the pH was around 7; in this condition the oxyrase (effective pH range is 6 to 9) could effectively remove the dissolved oxygen and keep an anaerobic condition; once the pH declined (< 6), its efficiency decreased gradually, but by that time it had helped the bacteria reach high cell densities which were more resistant to oxygen (14). When cells reached the stationary growth phase, because of acid accumulation they gradually lost viability. That is why media without internal or external pH control do not support growth well. In this experiment, shaken cultures always had lower cell populations than non-shaken cultures at the end of incubation. This is probably because both groups under the oxyrase system (0.15 unit/ml level) effectively maintain an anaerobic condition during early growth. However later in the incubation period (12 hr to 24 hr), oxyrase may be depleted and the increased dissolved oxygen allowed H_2O_2 or O_2^- formation which killed the organisms (14).

COMPARISON OF SODIUM GLYCEROPHOSPHATE AND TRIMAGNESIUM

PHOSPHATE

The superiority of pH-controlled lactic culture fermentations over conventionally produced batch cultures is well documented (26,27). Sandine and Ayres introduced the concept of bulk culture preparation using internal pH control medium (26). Since a buffered system is necessary for oxyrase to function well and to neutralize the acid produced by bifidobacteria, two different kinds of internal pH-controlled agents were added. Under a 20 rpm agitation condition, the 0.03 unit/ml of oxyrase could not efficiently remove the continuously dissolved oxygen, and the cells lost viability quickly. In contrast, in medium without any agitation viability was maintained throughout the incubation time, and the final populations were much higher than in the same medium without internal pH-control; for example, strains 15696, 15700, 15707, and L10 were 5, 3, 2, and 7 times higher, respectively. The two neutralizers, SG and TP, were essentially equal in support of final cell densities attained. However during early incubation, the TP-containing medium had a higher cell density than the SG-containing medium before the 8 hr incubation in all of the 4 tested strains. The pH decline was more smooth in TP containing medium than in SG medium, which has a very strong buffer capacity between pH 6.5 and 7.0, while after 8 hours of incubation, the pH decrease was more rapid in SG-containing medium than in TP medium.

GROWTH FACTOR OF OXYRASE

Oxyrase, an E. coli membrane fraction, removes dissolved oxygen in the presence of hydrogen donors(1,2). Several papers have

reported the use of oxyrase for culturing anaerobes (1,2,6). However, no reports describe possible growth promoting effect for oxyrase. In this experiment boiled oxyrase (0.3 unit/ml) was incorporated in the WC medium (WCO-b). Only B. longum 15707 required the oxyrase as a growth stimulator. In this case a 1.0 x 10^8 CFU/ml was achieved after 24 hours of incubation, but the population was still lower than in the WCY medium, because the final pH of the WCO-b was about 5.2 and the pH of the WCY medium was around 4.0 to 4.3. The reason lactose could not be efficiently used was unknown, and this probably implied that lactose utilization in strain 15707 was related to some growth factor from other sources (such as from YE). This also confirmed the above experiments that B. longum 15707 is more nutrient-dependent than dependent on an oxygen free condition. Here it is confirmed that most strains can grow in WC medium in a reduced condition, except the B. longum 15707, which needs some promoter for maximum growth.

GROWTH COMPARISON IN EVAPORATED MILK WITH MALT EXTRACT OR YEAST EXTRACT

A culture medium with malt extract and skim milk has been used to grow the B. longum under a nitrogen atmosphere to achieve high populations (17). However data shown in our experiment did not confirm this report. We tested 4 strains, including one strain (15707) which was claimed to reach high population densities in the ME-skim milk medium, and none of them showed vigorous growth in ME medium. YE still is the best growth factor in our studies, and even evaporated milk can not support the nutrient requirement for

most of the strains to grow. The reason we could not duplicate the previously-reported results is unknown but possibly was due to the varations in components present in different brands of malt extract.

GROWTH IN WHEY-BASED MEDIUM USING CULTURE PREPARED AS FROZEN CONCENTRATES

Concentrated starter cultures of lactic acid bacteria have proven easier to utilize in the manufacture of cultured products than traditional forms of starters (10). The advantages of using concentrated starters have been reported in the literature. In our studies, activated strains have been used exclusively. This was important because it has been reported that actively growing anaerobes are more resistant to oxygen (18) and high density population of active cells were able to overcome the toxicity of oxygenated medium, whereas populations of low density were unable to do so unless aided by reducing agents (14,18). Our findings indicate that a low inoculum from frozen concentrates had a lengthy lag phase before active growth occurred. Bifidobacterium longum L10, with an initial inoculum of 5 x 10^6 CFU/ml, revealed slow growth in medium without oxyrase, while vigorous growth occurred in medium with oxyrase. Also B. bifidum 15696 grew even better in medium with oxyrase, indicating this strain was more sensitive to oxygen. The other strains with inocula of 10^7 to 10^8 CFU/ml did not reveal any growth differences in these two media, confirming that in high population densities they were able to overcome the oxygen-damaging effect.

STABILITY OF BIFIDOBACTERIA IN REFRIGERATED MILK AND AS FROZEN CONCENTRATES

The role of microorganisms as dietary adjuncts has been considered since Metchnikoff first suggested the desirability of humans consuming lactobacilli capable of living in the intestinal tract (16). Continuous world-wide interest in the potential benefits of lactobacilli/bifidobacteria as dietary adjuncts has resulted in extensive research which has led to the development of dairy foods, pharmaceutical drugs and animal additives. Recently, the importance of maintaining a balance among beneficial microorganisms naturally present in the intestines was recognized, and several researchers reported that the bifidobacteria were especially important (13,21). In this regard, cultures of bifidobacteria have been added to infant food preparations to modify the intestinal flora and serve as a growth promoting additive (13). Products produced in Japan and European countries contain mainly bacteria of the Bifidobacterium and/or Lactobacillus genera. In the United States, extensive research has concerned the lactobacilli, but studies on bifidobacteria are limited (13). To use microorganisms as dietary adjuncts, maintainence of viability and metabolic activity in the carrier food before consumption is necessary, and the daily dosage level must be sufficiently high. For this it has been suggested that 10^8 to 10^9 viable microorganisms be consumed per day (11). In our results, freshly isolated bifidobacteria from infant stool samples were not stable in refrigerated milk; for example, B. breve T10, B. longum L10, B. longum L12, and B. bifidum L6 lost 99%, 80%, 70%, and 60% of viability, respectively, in the first 10 days of storage at 4°C in 11%

skim milk. Storage in milk with cysteine enhanced stability and strains from A.T.C.C. were more stable than those freshly isolated from baby feces. Strains15700, 15707, and 15698 did not lose viability when stored in 0.05% cysteine skim milk, but lost about 60% to 80% viability in skim milk only.

Early reports on frozen concentrated cultures of lactic streptococci (lactococci) frozen at pH 7 revealed much less loss of viability during 9 months of storage at -20°C than those frozen at pH 5 (15). Thus the pH of cultures at freezing is an important factor in governing how well cultures survive during frozen storage. Reports on storage temperature indicate that survival response was strain dependent; some strains were stable at -20 to -30°C while others were not stable when frozen at these temperatures (15). There has been no clear explanation for these differences in storage stability, however it generally is accepted that temperatures of -20°C or above are most deleterious and for long term storage, the lower the better.

Over the years, many attempts have been made to add cryoprotective agents to starter cultures in order to improve their ability to survive freezing and frozen storage. In our studies, when 9 strains of Bifidobacterium were frozen at -40°C and stored for 6 months in WCY-0.3 (7% sweet whey, 0.05% cysteine, 0.3% yeast extract) supplemented with 15% glycerol, survival was strictly strain dependent. The medium and storage temperature did not cause any damage to 4 bifidobacterial strains from A.T.C.C., but those freshly isolated from baby feces lost about 90% viability, except for B. bifidum L6 and B. longum L12. It has been theorized that the presence of oleic acid in the growth medium makes the membrane of

the cells more flexible, thus preventing damage resulting from freezing and thawing (11). However, in these studies strict anaerobic conditions were not maintained, since the cultures were dispersed into tubes prior to freezing under aerobic conditions. Therefore, the causes of viability loss in this study, whether due to oxygen or freezing damage or other factors is still unknown.

Table 1.1: Growth characteristics of B. bifidum 15696 in whey-based medium (7% sweet whey, .05% cysteine plus different amount of yeast extract and oxyrase). Inoculum at time zero was 2 x10^6 CFU/ml.

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
0%Y [a]-0 [b]	6.38	9.0 x 10^5	6.31	<104	6.17	<104
0%Y-.03	5.40	4.0 x 10^8	4.81	1.5 x 10^9	4.49	6.9 x10^8
0%Y-.15	5.35	3.5 x 10^8	4.75	1.2 x 10^9	4.48	7.1 x 10^8
0.1%Y-0	6.10	1.0 x 10^7	5.18	2.6 x 10^7	4.48	2.2 x 10^8
0.1%Y-.03	4.70	5.0 x 10^8	4.34	1.9 x 10^9	4.13	8.1 x 10^8
0.1%Y-.15	4.70	6.0 x 10^8	4.33	1.4 x 10^9	4.16	9.0 x10^8
0.3%Y-0	5.05	1.5 x 10^8	4.49	4.8 x 10^8	4.22	1.1 x 10^9
0.3%Y-.03	4.70	7.0 x 10^8	4.33	1.4 x 10^9	4.15	7.5 x 10^8
0.3%Y-.15	4.70	7.0 x 10^8	4.32	1.2 x 10^9	4.14	4.6 x 10^8
0.5%Y-0	5.00	1.6 x 10^8	4.42	4.3 x 10^8	4.13	8.6 x 10^8
0.5%Y-.03	4.72	5.2 x 10^8	4.33	1.7 x 10^9	4.15	8.0 x 10^8
0.5%Y-.15	4.70	6.5 x 10^8	4.33	9.2 x 10^8	4.15	5.1 x 10^8

[a] percentage of yeast extract incorporated into the whey-based medium.

[b] oxyrase unit per ml of whey-based medium (oxyrase purchased at 30 units per ml).

Table 1.2: Growth characteristics of B. breve 15700 in whey-based medium (7% sweet whey, .05% cysteine plus different amounts of yeast extract and oxyrase). Inoculum at time zero was 1 x 10^7 CFU/ml.

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
0%Y [a] 0 [b]	6.40	1.0 x 107	6.31	4.8 x 106	6.17	<104
0%Y-.03	5.40	8.0 x 10^8	4.81	2.6 x 10^9	4.49	4.1 x 10^8
O%Y-.15	5.37	8.2 x 10^8	4.75	1.5 x 10^9	4.48	3.9 x 10^8
0.1%Y-0	6.06	8.0 x 10^7	5.18	7.0 x 10^8	4.48	1.6 x 10^9
0.1%Y-.03	4.78	1.0 x 10^9	4.34	5.8 x 10^9	4.13	8.8 x 10^8
0.1%Y-.15	4.70	1.0 x 10^9	4.33	4.4 x 10^9	4.16	8.0 x 10^8
0.3%Y-0	5.31	8.4 x 10^8	4.49	5.4 x 10^9	4.22	2.0 x 10^9
0.3%Y-.03	4.70	1.5 x 10^9	4.33	3.9 x 10^9	4.15	4.9 x 10^8
0.3%Y-.15	4.70	1.8 x 10^9	4.32	5.9 x 10^9	4.14	4.4 x 10^8
0.5%Y-0	4.82	9.0 x 10^8	4.42	7.8 x 10^9	4.13	1.7 x 10^9
0.5%Y-.03	4.65	2.0 x 10^9	4.33	5.0 x 10^9	4.15	7.0 x 10^8
0.5%Y-.15	4.67	2.1 x 10^9	4.33	3.6 x 10^9	4.15	5.0 x 10^8

[a] percentage of yeast extract incorporated into whey-based medium.

[b] oxyrase units per ml of whey-based medium (oxyrase purchased at 30 units per ml).

Table 1.3: Growth characteristics of B. longum 15707 in whey-based medium (7% sweet whey, .05% cysteine plus different amounts of yeast extract and oxyrase). Inoculum at time zero was 4 x 10^6 CFU/ml.

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
0%Y [a]-0 [b]	6.60	1.0 x 10^6	6.61	6.8 x 10^5	6.41	2.7 x 10^5
0%Y-.03	5.95	2.0 x 10^8	5.29	2.6 x 10^8	4.63	2.9 x 10^8
0%Y-.15	5.70	3.1 x 10^8	4.89	3.8 x 10^8	4.39	3.8 x 10^8
0.1%Y-0	6.50	3.0 x 10^6	6.20	4.5 x 10^7	5.09	1.5 x 10^8
0.1%Y-.03	5.51	8.0 x 10^8	4.63	1.0 x 10^9	4.09	5.0 x 10^7
0.1%Y-.15	5.30	8.5 x 10^8	4.45	1.1 x 10^9	4.03	5.3 x 10^7
0.3%Y-0	5.80	4.1 x 10^8	4.79	9.1 x 10^8	4.15	1.1 x 10^9
0.3%Y-.03	5.34	8.5 x 10^8	4.44	1.6 x 10^9	3.95	1.2 x 10^8
0.3%Y-.15	5.21	8.0 x 10^8	4.33	1.7 x 10^9	3.92	1.3 x 10^8
0.5%Y-0	6.00	1.1 x 10^8	4.64	1.8 x 10^9	3.98	1.4 x 10^9
0.5%Y-.03	5.34	8.6 x 10^8	4.36	3.8 x 10^9	3.90	2.5 x 10^8
0.5%Y-.15	5.20	8.5 x 10^8	4.35	2.0 x 10^9	3.93	1.3 x 10^8

[a] percentage of yeast extract incorporated into whey-based medium.

[b] oxyrase units per ml of whey-based medium (oxyrase purchased at 30 units per ml).

Table 1.4: Growth characteristics of B. longum L10 in whey-based medium(7% sweet whey, .05% cysteine plus different amounts of yeast extract and oxyrase). Inoculum at time zero was 3 x 10^6 CFU/ml.

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
0%Y [a]-0 [b]	6.40	4.0 x 10^6	6.29	5.2 x 10^5	6.01	<10^4
0%Y-.03	5.70	4.0 x 10^8	4.70	1.0 x 10^9	4.14	4.6 x 10^8
0%Y-.15	5.50	8.0 x 10^8	4.67	1.1 x 10^9	4.16	5.1 x 10^8
0.1%Y-0	6.35	6.0 x 10^6	6.09	1.0 x 10^7	4.39	3.9 x 10^8
0.1%Y-.03	5.31	8.5 x 10^8	4.22	1.2 x 10^9	3.88	1.1 x 10^8
0.1%Y-.15	5.30	8.5 x 10^8	4.23	1.2 x 10^9	3.89	8.3 x 10^7
0.3%Y-0	5.80	1.0 x 10^8	4.35	1.1 x 10^9	3.81	1.7 x 10^9
0.3%Y-.03	5.30	8.6 x 10^8	4.19	1.1 x 10^9	3.82	1.0 x 10^9
0.3%Y-.15	5.29	8.7 x 10^8	4.23	1.0 x 10^9	3.85	1.1 x 10^9
0.5%Y-0	5.75	2.2 x 10^8	4.31	2.1 x 10^9	3.83	1.8 x 10^9
0.5%Y-.03	5.30	8.6 x 10^8	4.18	1.2 x 10^9	3.82	1.1 x 10^9
0.5%Y-.15	5.28	8.1 x 10^8	4.21	1.1 x 10^9	3.85	7.4 x 10^8

[a] percentage of yeast extract incorporated into whey-based medium.

[b] oxyrase units per ml of whey-based medium (oxyrase purchased at 30 units per ml).

Table 1.5: Growth characteristics of B. breve 15698 in whey-based medium (7% sweet whey, .05% cysteine plus different amounts of yeast extract and oxyrase). Inoculum at time zero was 1 x 10^7CFU/ml.

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
0%Y [a]-0 [b]	6.29	1.5 x 10^7	6.16	7.0 x 10^6	5.64	4.2 x 10^7
0%Y-.03	5.94	6.4 x 10^8	4.97	1.0 x 10^9	4.24	1.6 x 10^9
0%Y-.15	5.79	7.8 x 10^8	4.83	1.3 x 10^9	4.32	8.0 x10^8
0.1%Y-0	6.23	9.0 x 10^7	4.69	1.3 x 10^9	3.90	3.6 x 10^9
0.1%Y-.03	5.80	9.5 x 10^8	4.17	2.2 x 10^9	3.81	3.2 x 10^9
0.1%Y-.15	5.76	1.1 x 10^9	4.22	1.5 x 10^9	3.87	1.4 x 10^9
0.3%Y-0	6.00	5.4 x 10^8	4.25	3.0 x 10^9	3.82	4.9 x 10^9
0.3%Y-.03	5.68	1.2 x 10^9	4.14	2.0 x 10^9	3.79	2.9 x 10^9
0.3%Y-.15	5.65	1.2 x 10^9	4.18	1.8 x 10^9	3.85	8.9 x 10^8
0.5%Y-0	5.96	7.5 x 10^8	4.19	6.5 x 10^9	3.81	7.3 x 10^9
0.5%Y-.03	5.80	9.0 x 10^8	4.14	4.7 x 10^9	3.79	3.3 x 10^9
0.5%Y-.15	5.62	1.4 x 10^9	4.18	3.3 x 10^9	3.85	9.0 x 10^9

[a] percentage of yeast extract incorporated into whey-based medium.

[b] oxyrase units per ml of whey-based medium (oxyrase purchased at 30 units per ml)

Table 1.6: Growth characteristics of B. longum L12 in whey-based medium(7% sweet whey, .05% cysteine plus different amounts of yeast extract and oxyrase). Inoculum at time zero was 1x 10^6 CFU/ml.

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
0%Y [a]-0 [b]	6.45	3.0 x 10^6	6.33	4.2 x 10^4	6.17	< 10^4
0%Y-.03	6.38	2.0 x 10^7	5.07	8.0 x 10^8	4.37	3.2 x 10^8
0%Y-.15	6.29	3.3 x 10^7	4.96	7.3 x 10^8	4.44	4.1 x 10^8
0.1%Y-0	6.45	4.0 x 10^6	6.21	1.0 x 10^7	4.40	5.9 x 10^8
0.1%Y-.03	5.80	2.0 x 10^8	4.22	3.0 x 10^9	3.90	8.5 x 10^8
0.1%Y-.15	5.57	4.1 x 10^8	4.21	3.1 x 10^9	3.94	4.5 x 10^8
0.3%Y-0	6.28	1.6 x 10^7	4.43	1.2 x 10^9	3.86	1.0 x 10^9
0.3%Y-.03	5.67	1.3 x 10^8	4.13	4.0 x 10^9	3.81	1.0 x 10^9
0.3%Y-.15	5.53	1.6 x 10^8	4.15	1.3 x 10^9	3.83	5.3 x 10^8
0.5%Y-0	6.11	5.0 x 10^7	4.21	9.3 x 10^8	3.84	8.0 x 10^8
0.5%Y-.03	5.73	1.7 x 10^8	4.12	8.3 x 10^8	3.82	3.7 x 10^8
0.5%Y-.15	5.59	1.9 x 10^8	4.14	1.0 x 10^9	3.82	5.0 x 10^8

[a] percentage of yeast extract incorporated into whey-based medium.

[b] oxyrase unit per ml of whey-based medium (oxyrase purchased at 30 units per ml).

Table 1.7: Growth characterictics of B. longum 3j in whey-based medium (7% sweet whey, .05% cysteine, plus different amounts of yeast extract and oxyrase). Inoculum at time zero was 1.8 x 10^6 CFU/ml.

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
0%Y [a]-0 [b]	6.38	1.2 x 107	6.28	3.1 x 107	4.92	1.8 x 108
0%Y-.03	6.34	2.1 x 107	5.09	5.8 x 108	4.29	2.8 x 108
0%Y-.15	6.30	2.6 x 107	5.15	6.9 x 108	4.46	2.8 x 108
0.1%Y-0	6.42	1.2 x 107	5.36	4.4 x 108	3.89	6.1 x 107
0.1%Y-.03	6.38	3.6 x 107	4.72	5.3 x 108	3.88	1.4 x 107
0.1%Y-.15	6.34	6.0 x 107	4.71	5.7 x 108	3.91	1.1 x 107
0.3%Y-0	6.37	4.7 x 107	4.81	5.3 x 108	3.78	2.5 x 108
0.3%Y-.03	6.38	3.0 x 107	4.69	6.7 x 108	3.81	2.6 x 107
0.3%Y-.15	6.34	4.3 x 107	4.68	3.1 x 108	3.90	4.4 x 107
0.5%Y-0	6.44	2.6 x 107	4.78	4.4 x 108	3.75	7.4 x 107
0.5%Y-.03	6.44	2.9 x 107	4.70	6.3 x 108	3.79	1.8 x 107
0.5%Y-.15	6.40	4.1 x 107	4.70	6.0 x 108	3.87	1.5 x 107

[a] percentage of yeast extract incorporated into whey-based medium.

[b] oxyrase units per ml of whey-based medium (oxyrase purchased at 30 units per ml).

Table 1.8: Growth characteristics of B. bifidum 15696, B. breve 15700, and B. longum15707 in 7% sweet whey plus 0.3% yeast extract containing 0.15 unit/ml oxyrase with or without cysteine(C), and/or disodium glycerophosphate(D); (without extra agitation, but losely capped)

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
15696 C^-D^-	5.90	5.0×10^8	4.69	4.3×10^8	4.17	9.1×10^7
15696 C^-D^+	6.71	2.1×10^8	6.07	1.1×10^9	4.66	7.3×10^8
15696 C^+D^-	5.82	3.0×10^8	4.50	1.1×10^9	4.02	4.3×10^8
15696 C^+D^+	6.70	2.1×10^8	4.97	2.3×10^9	4.14	2.3×10^9
15700 C^-D^-	5.50	9.1×10^8	4.53	3.0×10^9	4.37	5.8×10^7
15700 C^-D^+	6.71	8.8×10^8	5.56	1.0×10^{10}	4.72	1.0×10^{10}
15700 C^+D^-	5.41	9.0×10^8	4.53	3.0×10^9	4.35	5.3×10^7
15700 C^+D^+	6.67	8.8×10^8	4.87	8.1×10^9	4.62	4.0×10^9
15707 C^-D^-	5.77	8.5×10^8	4.64	2.5×10^9	4.06	6.7×10^8
15707 C^-D^+	6.72	5.1×10^8	5.89	2.6×10^9	4.31	4.6×10^9
15707 C^+D^-	5.61	8.6×10^8	4.52	2.0×10^9	3.95	1.3×10^7
15707 C^+D^+	6.70	4.1×10^8	5.24	3.5×10^9	4.19	1.8×10^9

Time zero inoculum : 15696 (1.0×10^6 CFU/ml); 15700 (1.4×10^7 CFU/ml); 15707 (3.4×10^6 CFU/ml).

Table 1.8: continued

C^-D^-: medium without any cysteine or disodium glycerophosphate.

C^-D^+: medium without cysteine, but with 1.9% disodium glycerophosphate.

C^+D^-: medium without disodium glycerophosphate, but with .05% cysteine.

C^+D^+: medium contained both .05% cysteine and 1.9% disodium glycerophosphate.

Table 1.9: Growth characteristics of B. bifidum 15696, B. breve 15700, and B. longum 15707 in 7% sweet whey plus 0.3% yeast extract containing 0.15 unit/ml oxyrase with or without cysteine(C), and/or disodium glycerophosphate(D); (losely capped, under 100 rpm shaking condition).

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
15696 C^-D^-	5.4	5.0×10^8	4.53	6.3×10^8	4.10	1.3×10^8
15696 C^-D^+	6.35	4.5×10^8	5.74	5.9×10^8	4.55	4.6×10^8
15696 C^+D^-	5.32	5.1×10^8	4.27	8.4×10^8	3.98	1.4×10^8
15696 C^+D^+	6.20	7.3×10^8	4.59	1.7×10^9	4.08	4.6×10^8
15700 C^-D^-	5.30	8.5×10^8	4.53	1.7×10^9	4.35	1.7×10^8
15700 C^-D^+	6.42	8.3×10^8	5.54	3.3×10^9	4.67	5.0×10^9
15700 C^+D^-	5.02	9.2×10^8	4.43	2.0×10^9	4.31	9.7×10^7
15700 C^+D^+	5.80	1.0×10^9	4.74	8.9×10^9	4.62	7.1×10^8
15707 C^-D^-	5.31	5.1×10^8	4.56	1.0×10^9	4.02	6.0×10^8
15707 C^-D^+	6.48	4.5×10^8	5.69	7.1×10^8	4.37	1.6×10^9
15707 C^+D^-	5.24	3.6×10^8	4.39	7.9×10^8	3.91	7.8×10^7
15707 C^+D^+	6.21	4.1×10^8	5.01	1.3×10^9	4.18	1.3×10^9

Time zero inoculum: 15696 (1.9×10^6 CFU/ml); 15700 (7.6×10^6 CFU/ml); 15707 (3.6×10^6 CFU/ml).

Table 1.9: continued

C^-D^-: medium without any cysteine or disodium glycerophosphate.

C^-D^+: medium without cysteine, but with 1.9% disodium glycerophosphate.

C^+D^-: medium without disodium glycerophosphate, but with 0.05% cysteine.

C^+D^+: medium containing both 0.05% cysteine and 1.9% disodium glycerophosphate.

Table 1.10: Growth characteristics of B. bifidum 15696, B. breve 15700, B. longum 15707, and B. longum L10 in WCYO[a] with one of two different internal pH-control neutralizers with agitation at 20 rpm.

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
15696 SG[b]	6.80	3.1×10^5	6.77	$< 10^5$	6.06	$< 10^4$
15696 MP[c]	6.80	6.1×10^5	6.80	$< 10^5$	6.12	$< 10^4$
15700 SG	6.83	2.3×10^6	6.81	$< 10^5$	6.01	$< 10^4$
15700 MP	6.82	1.3×10^6	6.82	$< 10^5$	6.03	$< 10^4$
15707 SG	6.84	5.3×10^5	6.82	$< 10^5$	6.04	$< 10^4$
15707 MP	6.82	6.2×10^5	6.79	$< 10^5$	5.98	$< 10^4$
L10 SG	6.84	1.5×10^5	6.81	$< 10^5$	5.97	$< 10^4$
L10 MP	6.85	4.0×10^5	6.84	$<10^5$	6.05	$< 10^4$

Time zero inoculum: 15696 (1.4×10^6 CFU/ml); 15700 (9.0×10^6 CFU/ml); 15707 (2.8×10^6 CFU/ml); L10 (3.4×10^6 CFU/ml)

Table 1.10 continued

[a]WCYO: 7% sweet whey, 0.05% cysteine, 0.3% yeast extract, and 0.03 unit/ml oxyrase.

[b]SG: 1.9% disodium glycerophosphate

[c]MP: 1.9% trimagnesium phosphate

Table 1.11: Growth characteristics of B. bifidum 15696, B. breve 15700, B. longum 15707, and B. longum L10 in WCYO [a] with one of two different internal pH-control neutralizers (without any agitation).

	6hr		12hr		24hr	
medium	pH	CFU	pH	CFU	pH	CFU
15696 SG[b]	6.61	4.1×10^8	5.01	6.6×10^9	4.12	6.9×10^9
15696 MP[c]	6.52	9.0×10^8	5.67	5.2×10^9	4.60	6.6×10^9
15700 SG	6.59	8.1×10^8	4.94	1.7×10^{10}	4.59	1.0×10^{10}
15700 MP	6.51	9.0×10^8	5.64	1.2×10^{10}	5.02	1.9×10^{10}
15707 SG	6.72	2.5×10^8	5.86	2.6×10^9	4.54	2.3×10^9
15707 MP	6.64	5.4×10^8	5.92	2.9×10^9	5.46	3.1×10^9
L10 SG	6.70	3.2×10^8	4.64	9.4×10^9	4.00	7.0×10^9
L10 MP	6.40	1.0×10^9	5.47	1.4×10^{10}	4.25	1.2×10^{10}

Time zero inoculum: 15696 (1.9×10^6 CFU/ml); 15700 (1.0×10^7 CFU/ml); 15707 (3.2×10^6 CFU/ml); L10 (3.6×10^6 CFU/ml).

[a]WCYO: 7% sweet whey, 0.05% cysteine, 0.3% yeast extract, and 0.03 unit/ml oxyrase.

[b]SG: 1.9% disodium glycerophosphate

[c]MP: 1.9% trimagnesium phosphate

Table 1.12: Growth characteristics of B. bifidum 15696, B. breve 15700, B. longum 15707, and B. breve 15698 in evaporated milk(e) with incorporated yeast extract(Y) or malt extract(M).

	12hr		24hr		36hr		48hr	
medium	pH	CFU	pH	CFU	pH	CFU	pH	CFU
15696e	6.13	1.0×10^6	6.10	4.0×10^5	6.03	7.0×10^4	6.10	3.0×10^3
15696eY	5.17	1.0×10^8	4.98	1.2×10^8	4.80	8.0×10^7	4.91	5.8×10^7
15696eM	6.15	1.7×10^6	6.13	6.0×10^6	5.54	1.4×10^8	5.33	1.0×10^8
15700e	6.04	7.7×10^7	5.75	9.2×10^7	5.50	1.0×10^6	5.56	4.1×10^5
15700eY	5.31	6.7×10^8	4.72	2.7×10^9	4.60	7.2×10^8	4.67	2.7×10^7
15700eM	6.15	1.0×10^6	6.10	2.0×10^7	5.81	1.5×10^8	5.62	9.2×10^7
15707e	6.16	5.0×10^6	6.13	6.0×10^5	6.06	4.0×10^4	6.08	3.0×10^2
15707eY	5.28	5.3×10^8	4.67	2.5×10^9	4.31	1.3×10^{99}	4.20	6.7×10^8
15707eM	6.15	6.0×10^6	5.97	7.0×10^7	5.34	5.5×10^8	5.19	3.7×10^8
15698e	6.00	2.8×10^8	5.58	5.0×10^8	5.25	1.5×10^8	5.15	2.6×10^8
15698eY	5.21	3.2×10^9	4.50	5.3×10^9	4.11	7.3×10^9	4.04	7.5×10^9
15698eM	6.08	6.0×10^6	5.97	2.8×10^7	5.43	6.4×10^8	5.07	1.2×10^9

Time zero inoculum: 15696 (1.8×10^6 CFU/ml); 15700 (1.1×10^7 CFU/ml); 15707 (5.4×10^6 CFU/ml); 15698 (6.2×10^6 CFU/ml)

e: evaporated milk.

eY: evaporated milk with 0.3% yeast extract.

eM: evaporated milk with 10% malt extract.

Table 1.13: Oxyrase(o) growth factor assessment using 7% sweet whey, 0.05% cysteine as the basal medium(b) for culturing B. bifidum 15696, B. breve 15700, B. longum 15707, B. breve 15698 B. longum L10, B. longum L12.

	6hr		12hr		24hr	
	pH	CFU	pH	CFU	pH	CFU
15696b	6.40	1.0×10^6	6.29	8.0×10^6	4.42	8.1×10^8
15696bo	6.35	4.0×10^6	6.20	3.3×10^7	4.27	8.2×10^8
15700b	6.21	6.7×10^7	5.48	4.2×10^8	4.31	5.2×10^9
15700bo	6.14	1.2×10^8	5.14	1.2×10^9	4.27	6.0×10^9
15707b	6.43	6.0×10^6	6.37	$< 10^6$	6.24	$< 10^6$
15707bo	6.33	4.0×10^6	5.97	1.2×10^8	5.21	1.6×10^8
15698b	6.26	6.0×10^7	5.33	4.1×10^8	4.31	4.5×10^9
15698bo	6.25	4.0×10^7	5.20	6.3×10^8	4.22	4.8×10^9
L10b	6.36	5.0×10^6	5.80	2.2×10^8	4.07	2.7×10^9
L10bo	6.34	6.0×10^6	5.56	5.3×10^8	4.02	2.0×10^9
L12b	6.38	7.0×10^6	5.93	5.0×10^7	4.30	1.0×10^9
L12bo	6.33	6.6×10^6	5.82	7.0×10^7	4.13	1.2×10^9

Time zero inoculum: 15696 (1.4×10^6 CFU/ml); 15700 (1.5×10^7 CFU/ml); 15707 (2.5×10^6 CFU/ml); 15698 (1.4×10^7 CFU/ml); L10 (3.0×10^6 CFU/ml); L12 (2.0×10^6 CFU/ml).

b. : basal medium ; bo : basal medium with 0.3 units/ml boiled (100° C,10 min) oxyrase.

Table 1.14: Growth characteristics of strains measured directly from frozen concentrates in WCY medium with or without oxyrase..

	6hr		12hr		24hr	
	pH	CFU	pH	CFU	pH	CFU
15696WCY	6.35	5.0×10^7	4.81	3.0×10^8	3.92	5.9×10^8
15696WCYO	6.30	6.6×10^7	4.38	2.0×10^9	3.71	6.2×10^8
15700WCY	6.22	3.8×10^8	4.45	4.0×10^9	3.83	3.9×10^9
15700WCYO	6.16	2.1×10^8	4.39	4.0×10^9	3.81	3.6×10^9
15707WCY	6.42	8.2×10^6	5.11	7.8×10^8	3.98	1.4×10^9
15707WCYO	6.39	1.3×10^7	4.83	8.0×10^8	3.80	9.5×10^8
15698WCY	6.12	3.8×10^8	4.15	4.0×10^9	3.61	4.4×10^9
15698WCYO	5.91	4.9×10^8	4.05	6.0×10^9	3.57	4.9×10^9
L10WCY	6.43	5.3×10^6	5.97	6.6×10^7	3.63	5.2×10^9
L10WCYO	6.38	2.0×10^7	4.44	2.1×10^9	3.52	1.7×10^9
T10WCY	5.91	2.8×10^8	4.02	4.0×10^9	3.50	3.6×10^9
T10WCYO	5.64	7.7×10^8	3.94	6.0×10^9	3.52	4.9×10^9

Time zero inoculum: 15696 (1.5×10^7 CFU/ml); 15700 (8.0×10^7 CFU/ml); 15707 (1.2×10^7 CFU/ml); 15698 (1.5×10^8 CFU/ml); L10 (5.0×10^6 CFU/ml); T10 (8.0×10^7 CFU/ml).

WCY: 7% sweet whey, 0.05% cysteine, and 0.3% yeast extract.

WCYO: WCY with 0.03 unit/ml oxyrase.

Table 1.15: Stability of bifidobacteria grown in whey-based medium and stored in refrigerated milk with or without cysteine for 40 days.

	15696		15700		15707		L10	
Days	cys^+	cys^-	cys^+	cys^-	cys^+	cys^-	cys^+	cys^-
0	$3.8x10^9$	$4.2x10^9$	$1.3x10^{10}$	$8.8x10^9$	$1.3x10^9$	$1.0x10^9$	$2.0x10^9$	$1.6x10^9$
2	$2.2x10^9$	$1.7x10^9$	$1.0x10^{10}$	$1.1x10^{10}$	$1.6x10^9$	$8.0x10^8$	$2.5x10^9$	$5.0x10^8$
4	$3.1x10^9$	$1.0x10^9$	$1.0x10^{10}$	$7.6x10^9$	$1.4x10^9$	$9.0x10^8$	$1.3x10^9$	$8.1x10^8$
6	$1.7x10^9$	$9.0x10^8$	$1.3x10^{10}$	$8.8x10^9$	$1.6x10^9$	$5.0x10^8$	$1.3x10^9$	$9.5x10^8$
8	$1.2x10^9$	$4.7x10^8$	$1.2x10^{10}$	$3.2x10^9$	$1.6x10^9$	$3.0x10^8$	$1.0x10^9$	$7.0x10^8$
10	$9.8x10^8$	$7.0x10^8$	$1.3x10^{10}$	$1.7x10^9$	$1.4x10^9$	$3.4x10^8$	$9.1x10^8$	$2.6x10^8$
20	$1.6x10^8$	$2.0x10^7$	$5.0x10^9$	$1.0x10^9$	$1.0x10^9$	$1.6x10^8$	$4.7x10^8$	$1.1x108^8$
40	$5.1x10^7$	$< 10^6$	$6.0x10^9$	$8.0x10^8$	$5.0x10^8$	$1.0x10^8$	$8.1x10^7$	$7.0x10^7$

cys^+ : 11% skim milk with 0.05% cysteine.

cys^- : 11% skim milk only.

Table 1.15: continued

	15698		L6		L12		T10	
days	cys+	cys-	cys+	cys-	cys+	cys-	cys+	cys-
0	1.0×10^{10}	1.5×10^{10}	5.0×10^{8}	3.1×10^{9}	2.8×10^{9}	3.0×10^{9}	1.0×10^{10}	8.2×10^{9}
2	1.3×10^{10}	2.0×10^{10}	7.0×10^{8}	5.0×10^{9}	3.2×10^{9}	4.0×10^{9}	1.3×10^{10}	7.8×10^{9}
4	9.0×10^{8}	1.0×10^{10}	6.1×10^{8}	3.5×10^{9}	2.6×10^{9}	2.0×10^{9}	3.1×10^{9}	6.0×10^{9}
6	1.0×10^{10}	9.4×10^{9}	7.0×10^{8}	1.4×10^{9}	7.0×10^{8}	1.0×10^{9}	3.0×10^{9}	7.0×10^{9}
8	8.0×10^{8}	7.0×10^{9}	6.3×10^{8}	1.7×10^{9}	7.0×10^{8}	1.5×10^{9}	1.0×10^{9}	8.0×10^{8}
10	1.5×10^{10}	6.0×10^{9}	6.8×10^{8}	1.6×10^{9}	4.0×10^{8}	8.0×10^{8}	2.0×10^{8}	1.0×10^{8}
20	5.0×10^{9}	3.0×10^{9}	6.0×10^{8}	8.0×10^{8}	1.0×10^{8}	9.0×10^{7}	1.1×10^{8}	3.0×10^{8}
40	1.0×10^{9}	1.6×10^{9}	1.0×10^{8}	1.0×10^{8}	2.0×10^{7}	$< 10^{6}$	5.0×10^{7}	1.0×10^{7}

cys+ : 11% skim milk with 0.05% cysteine.

cys- : 11% skim milk only.

Table 1.16: Stability of bifidobacteria as frozen concentrates[a] at -40 °C during 6 months of storage.

	peroids of storage					
strains	0 day	1 month	2-month	3-mont	4-month	6-month
15696	5.0×10^9	6.0×10^9	4.5×10^9	7.0×10^9	6.0×10^9	6.0×10^9
15700	2.5×10^{10}	1.8×10^{10}	1.5×10^{10}	2.0×10^{10}	1.5×10^{10}	1.0×10^{10}
15707	2.5×10^9	1.8×10^9	2.7×10^9	3.0×10^9	1.9×10^9	2.1×10^9
15698	3.0×10^{10}	2.5×10^{10}	3.4×10^{10}	3.0×10^{10}	2.6×10^{10}	2.0×10^{10}
L6	1.0×10^9	5.0×10^8	5.1×10^8	6.3×10^8	4.0×10^8	6.0×10^8
L10	4.4×10^9	1.0×10^9	1.0×10^9	8.0×10^8	6.0×10^8	5.0×10^8
L12	5.0×10^9	6.0×10^9	8.0×10^9	6.0×10^9	4.0×10^9	3.0×10^9
T2	2.0×10^{10}	1.7×10^{10}	1.2×10^{10}	2.0×10^{10}	5.0×10^9	3.0×10^9
T10	2.3×10^{10}	1.9×10^{10}	1.7×10^{10}	1.0×10^{10}	6.0×10^9	1.0×10^9

[a] whey-based grown bifidobacteria suspended into 7% sweet whey containing 0.3% yeast extract, 0.05% cysteine and 15% glycerol.

REFERENCES

1. Adler, H. I., and W. D. Crow. 1981. A novel approach to the anaerobic microorganisms. Biotechnology and Bioengineering Symp. No. 11, 533-540.
2. Adler, H. I., W. D. Crow, C. T. Hadden, J. Hall, and R. Machanoff. 1983. New techniques for growth anaerobic bacteria: Experiments with Clostridium butyricum and Clostridium acetobutylicum. Biotechnology and Bioengineering Symp. No 13, 153-161.
3. Carlsson, J., G. Nyberg, and J. Wrethen. 1978. Hydrogen peroxide and superoxide radical formation in anaerobic broth media exposed to atmospheric oxygen. App. Enviro. Microbiol. 36: 223-229.
4. Carlsson, J., G. P. D. Granberg, G. K. Nyberg, and M. K. Edlund. 1979. Bactericidal effect of cysteine exposed to atmospheric oxygen. App. Enviro. Microbiol. 37: 383-390.
5. Collins, E. B., and B. J. Hall. 1984. Growth of bifidobacteria in milk and preparation of Bifidobacterium infantis for a dietary adjunct. J. Dairy Sci. 67: 1376-1380.
6. Crow, W. D., R. Machanoff, and H. I. Alder. 1985. Isolation of anaerobes using an oxygen reducing membrane fraction: experiments with acetone butanol producing organisms. J. Microbiol. Methods. 4:133-139.

7. Deguchi, Y., T. Morishita, and M. Mutai. 1985. Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. Agri. Biol. Chem. 49: 13-19.

8. Faure, J. C., D. Schellenberg, A. Bexter, and H. P. Wurzner. 1982. Barrier effect of Bifidobacterium on Escherichia coli in the germ-free rat. Int. J. Vitam. Nutr. Res. 52:225-230.

9. Ferrari, A. , N. Pacini, and E. Canzi. 1980. A note on bile acids transformations by strains of Bifidobacterium. J. Appl. Bacteriol. 49: 193-197.

10. Gilliland, S. E. 1977. The manufacture of starters by batch fermentation and centrifugation to produce concentrates. J. Soc. Dairy Technol. 30: 36-41.

11. Gilliland, S. E., M. L. Speck, G. F. Nauyok, and F. G. Giesbrecht. 1978. Influence of consuming nonfermented milk containing Lactobacillus acidophilus on fecal flora of healthy males. J. Dairy Sci. 61: 1-10.

12. Hoskins, C. B., E. Rico-Munoz, and P. M. Davidson. 1986. Aerobic incubation of Clostridium perfringens in the presence of an oxygen-reducing membrane fraction. J. Food Sci. 51: 1585-1586.

13. Kim, S. H. 1988. Characterization of Lactobacilli and Bifidobacterium as applied to dietary adjunts. Cult. Dairy Prod. J. 6-9.

14. Krieg, N. R., and P. S. Hoffman. 1986. Microaerophily and oxygen toxicity. Ann. Rev. Microbiol. 40: 107-130.

15. Lamprech, E. D., and E. M. Foster. 1963. The survival of starter

organisms in concentrated suspensions. J. Appl. Bacteriol. 26: 359- 363.

16. Metchnikoff, E. 1908. The prolongation of life. 1st edition. G. P. Putnams sons, New York.

17. Nagawa, M., A. Nakabayyashi, and S. Fujino. 1988. Preparation of the bifidus milk powder. J. Dairy Sci. 71: 1777-1782.

18. Patel, G. B., L. A. Roth, and B. J. Agnew. 1984. Death rates of obligate anaerobes exposed to oxygen and the effect of media prereduction on cell viability. Can. J. Microbiol. 30: 228-235.

19. Poch, M., and A. Bezkorovainy. 1988. Growth-enhancing supplements for various species of the Bifidobacterium. J. Dairy Sci. 71: 3214-3221

20. Poupard, J. A., L. Husain, and R. F. Norris. 1973. Biology of the bifidobacteria. Bacteriol. Rev. 37: 136-165.

21. Rasic, J. L., and J. A. Kurman. 1983. Bifidobacteria and their role. Library of congress cataloging in publication data.

22. Rolfe, R. D., D. J. Hentges, B. J. Campbell, and J. T. Barret. 1978. Factors related to the oxygen tolerence of anaerobic bacteria. App. Enviro. Microbiol. 36: 306-313.

23. Staab, J. A., and J. K. Ely. 1987. Viability of lyophilized anaerobes in two media. Cryobiology 24: 174-178.

24. Stark, P. L., and A. Lee. 1982. The microbial ecology of large bowel of breast-fed and formula-fed infants during the first year. J. Med. Microbiol. 15: 189-203.

25. Sykes, G., and F. A. Skinner. 1983. Techniques for the isolation and characterization of Actinomyces and Bifidobacterium

species: report of a panel discussion. Soc. Appl. Bact. Symp. Series No.2 p. 327-333.

26. Willrett, D. L., W. E. Sandine, and J. W. Ayres. 1979. Evaluation of a new pH-controlled bulk starter medium. Cheese reporter 103: 8-9

27. Wright, S. L., and G. H. Richardson. 1982. Optimization of whey-based or nonfat dry milk-based media for production of pH controlled bulk lactic cultures. J. Dairy Sci. 65: 1882-1889.

28. Yoshioka, H. Y., K.Iseki, and K. Fujita. 1983. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics 72: 317-321.

CHAPTER 2

CHARACTERIZATION OF BIFIDOBACTERIA ISOLATED FROM INFANT FECES

ABSTRACT

The fecal flora of 10 healthy infants aged 1 day to 4 months was examined for the presence of Bifidobacterium . Babies A, B, C, D, and E, were 1 to 5 days old and breast-fed; no bifidobacteria were recovered from their feces. Babies T and L were 15 days old and breast-fed, 40% and 50% of their fecal flora were identified as Bifidobacterium spp., respectively ; further characterization revealed that B. breve was dominant in baby T and B. longum was most prevalent in baby L. No bifidobacteria were found in bottle-fed baby W or baby H at 15 and 25 days old. From baby RC, a three-month-old breast-fed infant, 80% of the isolations were Bifidobacterium spp. ; further characterization showed these strains were atypical. Baby H was bottle-fed until 2 months old, then was breast-fed; after 2 months of breast-feeding, the fecal flora was characterized again, and 70% of the flora were Bifidobacterium spp.. In this study, it was observed that the percentage of Bifidobacterium spp. in the feces increased as the breast-feeding time increased. For example, 40% to 50% in 15-day-old baby L and T increased to 70% in 2-month old baby H and 80% in 3-month old baby RC.

INTRODUCTION

Normal gut microbial flora consists of microorganisms which live in a stable relationship with the host. Many factors control the size and composition of microbial populations in different regions of the gastrointestinal tract. The stomach and small intestine, particularly its upper part, contain few microorganisms, while in large intestine the microbial populations may be as high as 10^{10} to 10^{11}/gram (8,13). The gut bacteria have a high metabolic activity. They break down some dietary and endogenous compounds, undigested carbohydrate glucoside, biliary compounds and others; they also produce certain vitamins. Some products of bacterial metabolism may be a potential hazard to the health of the host.

It is generally accepted that morbidity and mortality are considerably lower in breast-fed infants than in bottle-fed infants (3,4). It also is well known that the nature of milk fed to newborn infants influences their physicochemical and microbiological intestinal condition; differences appear quickly between breast-fed and the bottle-fed infants. Breast-feeding induces liquid feces with a cheesy odor and an acid pH of 5.0. The feces of bottle-fed infants look like those of adults in consistency and odor and have a pH between 6.0 to 7.0 (6,7). It seems reasonable to suggest that the presence of lactoferrin and antibodies in colostrum, and early milk, plays an important role in the initial protective mechanism for infants against the multiplication of gram-negative organisms (13). This is due to the poor buffering capacity of breast milk (high lactose,

low protein and low phosphate content compared to lower lactose, higher phosphate and protein contents in cow's milk), which allow the pH level in the intestine to drop rapidly. The decreasing pH ultimately leads to conditions that are unfavorable for the growth of coliform organisms (8).

Since several genera of enteric bacteria bind preferentially to lactosylceramide in vitro, mucin-degrading bifidobacteria (such as B. infantis) likely play an important ecological role in host-microbial associations in the human gut (12). It is well known that the dominant microorganisms in the feces of breast-fed babies are bifidobacteria, while the fecal microflora of bottle-fed babies consists of various bacteria, i.e.. bifidobacteria, bacteroides, clostridia, enterobacteria, and streptococci (5,13). Despite a number of studies that have appeared in the literature related to the role of bacteria in intestinal health, the precise role they play is still speculative. The present investigation focused on identifying Bifidobacterium spp. isolated from the feces of babies and to determine if the type of feeding has any noticeable effect on the microbial composition of the feces.

MATERIALS AND METHODS

REAGENTS AND EQUIPMENT

Materials used and from where obtained: Sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, sodium iodoacetate, sodium fluoride, fructose-6-phosphate, trichloroacetic acid, cysteine, hydroxylamine-HCl, sodium hydroxide, amygdalin, glycogen, inositol, lactose, maltose, mannose, melezitose, raffinose, ribose, and sucrose were all obtained from Sigma Chemical Co., St. Louis, MD.

Oxyrase enzyme system was from Oxyrase Inc., Ashland, Ohio; Ferric chloride $6H_2O$ from Mallinckrodt; esculin from Nutritional Biochemicals Corporation. Arabinose, mannitol, sorbitol, and xylose were from J. T. Baker Chemical Co.. Reinforced Clostridial agar (RCA), and reinforced clostridial medium (RCM) were from Difco. GasPak anaerobic system was from BBL. The ultrasonic cleanser (model B-32 ,117 volts) was from Branson Company. Sterile cotton swabs were from Van Waters and Rogers, Ltd.

SUBJECTS

The study group consisted of 10 healthy babies aged from one day to four months (5 females and 5 males). They were born at Good Samaritan Hospital, Corvallis, Oregon, and delivered by the vaginal

route. None had a history of abnormal bowel function or of antibiotic therapy. Baby A and baby B (both males) were 1 day old. Baby C (female) was 2 days old. Baby D (male) was 3 days old. Baby E (male) was 5 days old. Baby T and baby L (both female) were 15 days old. All the above mentioned babies were breast-fed. Baby W (female) was 15 days old and bottle-fed. Baby RC (male) was 3 months old and breast-fed. Baby H (female) was bottle-fed before 2 months old then breast fed until 4 months old.

COLLECTION OF SPECIMENS

Fresh voided fecal samples were collected into sterile glass containers using sterile cotton swabs and placed in prereduced sterilized phosphate buffered saline solution containing 0.15 unit/ml of oxyrase.

CULTURE MEDIA AND METHODS

Specimens were cultured within one-half hour after collection. After through mixing, a series of 10-fold dilutions (10^{-1} to 10^{-9}) was made in phosphate-buffered saline solution (PBS,0.85% sodium chloride, 0.25% disodium phosphate, 0.056% sodium phosphate). From appropriate dilutions, 0.1-ml samples were pour-plated into non-selective RCA and incubated under anaerobic condition at 37°C in the GasPak system (BBL) for 48 hr. After incubation, plates (with 25 to 250 colonies) were selected and colonies (12 to 30 colonies) inoculated into 10 ml RCM incubated at 37°C for 12 hr. Cultures were

gram-stained and examined under the microscope; only Gram positive samples were further studied.

IDENTIFICATION OF ISOLATES

The identification of bifidobacteria was carried out by methods previously described by Scardovi (14), with slight modification. Cells harvested from 10 ml RCM were spun down at 6000 rpm for 10 min and washed twice using 0.05M phosphate buffer (pH 6.5 with 0.5 g/l cysteine) and resuspended in 1 ml of the same buffer. Cells were disrupted carefully by sonication (40 min) in a 4°C cold room then frozen and thawed twice. Sodium fluoride and sodium iodoacetate (0.25 ml each containing 6 mg/ml and10 mg/ml, respectively) and 0.25 ml sterile fructose-6-phosphate solution (70 mg/ml, membrane-filtered) were added and incubated at 37°C for 30 min. The reaction was stopped by adding 1.5 ml of hydroxylamine-HCl (13.9 g/ml, pH 6.5), freshly neutralized with 10N NaOH. After 10 min at room temperature, 1 ml each of trichloroacetic acid (15% wt/v) and 4N HCl followed by 1 ml of the color-developing reagent (ferric chloride $6H_2O$, 5% wt/v in 0.1N HCl) were added. Any reddish-violet color that immediately developed was taken as a positive result for bifidobacteria. Species determinations were carried out by sugar fermentation patterns using the following sugars; arabinose, xylose, ribose, mannose, sucrose, maltose, lactose, raffinose, melezitose, glycogen, mannitol, sorbitol, inositol, esculin, and amygdalin; 0.5% (w/v) of the sugars were incorporated into basal RCM (RCM without

beef extract, starch, dextrose, or agar) with a 2% inoculum of each strain tested (from 12 hour-cultures, spun down and resuspended in 10 ml PBS) and incubated at 37°C in GasPak system for 48 hr. Bifidobacterium spp. were defined according to Mitsuoka (11).

RESULTS

FECES FROM 1 TO 5 DAY OLD BREAST-FED INFANTS

Fourteen colonies were randomly picked from plated stools of each of babies A, B, C, D, and E, and tested according to Scardovi methodology (14). None of the colonies was identified as Bifidobacterium spp..

FECES FROM 15 DAY OLD BREAST-FED INFANTS

Twevle isolated colonies were tested from baby T and baby L. About 40% (5/12) and 50% (6/12), respectively, showed fructose-6-phosphate phosphoketolase (F6PPK) positive, a unique enzyme used by bifidobacteria in sugar fermentation. These strains were further characterized to species using sugar fermentation tests. Results are shown in Tables 2.1 to 2.2. Bifidobacterium longum were dominant in baby L and B. breve were dominant in baby T.

FECES FROM 15-DAY OLD BOTTLE-FED INFANT

A total of 20 colonies were isolated from the faeces of baby W and none of them were positive for F6PPK.

FECES FROM BOTTLE-FED INFANTS AT 15 AND 25 DAYS

Twenty four and thirty colonies were isolated from feces of baby H at 15 and 25 days, respectively and none of the colonies were F6PPK positive.

FECES FROM 3-MONTH OLD BREAST-FED INFANT

Twenty two colonies were randomly picked from plated feces of baby RC, about 80% (19/22) showing F6PPK positive results. These bacteria were further characterized by sugar fermentation ability. Data in Table 2.3 show that most of them were similar to B. longum, but also fermented a few other sugars. Bifidobacterium breve 15698 was used as a reference strain in this test to aid in identifying atypical strains.

FECES FROM A 4-MONTH OLD INFANT

Baby H was bottle-fed from up to 2 months old; during that time 2 samples taken at 15 and 25 days were tested and no bifidobacteria were found. Following 2 months, this baby was breast-fed until 5 months old. During the breast-feeding period, a sample was taken at 4 months, and 28 colonies were tested for F6PPK; about 70% (21/28) were positive. These strains were not furthrer characterized.

DISCUSSION

The purpose of this investigation was to test for the presence of Bifidobacterium in bottle- or breast-fed baby faeces. It is well known that E. coli, and other enterobacteria are sometime pathogens causing gastrointestinal infections in the newborn (4). It also has been repeatedly documented that the occurrence of enteropathogenic E. coli, Salmonella, and necrotizing enterocolitis are significantly higher in bottle-fed than in breast-fed infants. Although studies of microorganisms in the human gut involve greater variations in sample content and technique than studies in laboratory animals, certain patterns have become evident. Previous reports (4,5) suggested that in both groups, the intestine is first colonized with enterobacteria. Important differences appear quickly between breast-fed and bottle-fed infants. In breast-fed, enterobacteria decrease, and bifidobacteria increase, but in the bottle-fed group enterobacteria dominate in the intestines of the baby. By day 6, bifidobacteria are the dominant organisms in stools of breast-fed infants, whereas enterobacteria are still the dominant organisms in bottle-fed infants. Our findings are in accord with those observations, since in samples isolated from one- to five- day-old breast-fed babies, no bifidobacteria were recovered from the feces. At 15 day, the sequence of bacterial colonization was quite different in these two groups. In the breast-fed babies, bifidobacteria were easily isolated from the feces as compared to the bottle-fed babies. From samples taken at the same age (15 days) from baby T and baby L,

about 50% of colonies isolated from the highest dilution plate were bifidobacteria, but this was not the case for baby W or baby H. This was in contrast to an earlier study (3,4), where bifidobacteria were the most prevalent organisms in both feeding groups, but the number of bifidobacteria in the stool of bottle-fed infants was approximately one-tenth that of breast-fed infants at one month of age. In the present study, baby H was first bottle-fed for 2 month and then was breast fed until 5 months old. Fecal samples were taken at 15, 25 day and 4 months. During the bottle-feeding period, even though 24 and 30 colonies were tested at 15 and 25 days respectively, no bifidobacteria were recovered from the feces. After 2 months of bottle-feeding, the diet of baby H was changed back to breast milk and samples were picked at 4 months within the breast-feeding time. We found that 70% of the colonies were Bifidobacterium spp.. It is possible over the bottle-feeding period the bifidobacteria did exist, but were overwhelmmed by other bacteria; after the diet was changed back to human milk, growth promoters from the human milk stimulated the growth of bifidobacteria. Previous report (3) has considered the importance of bifidobacteria as " reflecting good health " or as a " protective agent itself ", since they are the dominant microorganisms in the intestines of breast-fed infants, while the fecal microflora of bottle-fed babies have a mixed biota. Our findings endorse this report that bifidobacteria are the prevalent bacteria in feces of breast-fed babies, especially since no Bifidobacterium spp. were recovered from the bottle-fed infants. Several bifidus growth factors from human milk have been reported in the literature (1,2) which could explain why bifidobacteria are the

dominant species. Why the Bifidobacterium were also found to be the dominant strain in earlier reports in the bottle-fed group is still unsolved; this may be partly due to improved baby formula.

Further characterization of Bifidobacterium. spp revealed that B. breve was the dominant strain in the intestines of baby T while B. longum was the prevalent strain in the feces of baby L; we did not isolate any B. infantis or B. adolescentis in this study, and strains characterized from baby RC were quite different from most of the Bifidobacterium found so far. According to Mitsuoka et al.(11), characteristics used to differentiate newly isolated species and biotypes within the genus Bifidobacterium are inconsistent with sugar fermentation patterns for known strains. It therefore has been suggested by Mitsuoka et al. (5), that further taxonomic studies of the Bifidobacterium group isolated from baby feces are necessary. These differences may be due to the complex intestinal environment of infants, changes in composition of breast milk or the intricate nature of formula milk. For the present study, an interesting result was observed: the percentage of bifidobacteria in the feces increased as the breast-feeding time increased, i.e. 40 to 50% of bifidobacteria in baby T and L (15 days) increased to 70% in baby H (2 months) and 80% in baby RC (3 months).

Table 2.1: Characterization of Bifidobacterium. spp T isolated from baby T (breast-fed,15 days old)

	strains				
Carbohydrate	T2	T7	T9	T10	T11
amygdalin	w	w	w	w	w
arabinose	-	-	-	-	-
esculin	w	w	w	-	w
glycogen	+	++	+	++	++
inositol	-	-	-	-	-
lactose	++	++	++	++	++
maltose	++	++	++	++	++
mannose	+	+	+	+	+
mannitol	+	+	+	+	+
melezitose	w	w	w	w	w
raffinose	+	+	+	+	+
ribose	-	+	+	+	+
sorbitol	+	+	+	+	+
sucrose	+	+	+	+	+
xylose	-	-	-	-	-

Symbol: ++ = strong reaction; + = positive reaction; w = weak reaction; - = negative reaction.

Table 2.2: Characterization of Bifidobacterium. spp. L isolated from baby L (breast-fed,15 days old)

	strains				
Carbohydrate	L1	L4	L6	L10	L12
amygdalin	-	-	-	-	-
arabinose	+	+	+	+	+
esculin	-	-	-	-	-
glycogen	-	-	-	-	-
inositol	-	-	-	-	-
lactose	++	++	++	++	++
maltose	++	++	++	++	++
mannose	+	+	+	+	+
mannitol	-	-	-	-	-
melezitose	+	+	-	+	+
raffinose	+	+	-	+	+
ribose	+	+	-	+	+
sorbitol	w	-	-	-	-
sucrose	+	+	-	+	+
xylose	+	+	+	+	+

Symbol: ++ = strong reaction; + = positive reaction; w = weak reaction; - = negative reaction.

Table 2.3: Characterization of Bifidobacterium. spp isolated from baby RC (breast-fed, 3 months old)

	Strains									
Carbohydrate	RC1	RC2	RC3	RC4	RC5	RC6	RC7	RC8	RC9	RC10
amygdalin	++	++	-	++	-	w	-	-	-	++
arabinose	+	+	-	+	-	-	-	-	-	+
esculin	-	-	-	-	-	w	-	-	-	-
glycogen	++	-	++	+	++	++	++	++	++	-
inositol	-	-	-	-	-	-	-	-	-	-
lactose	++	++	++	++	++	++	++	++	++	++
maltose	++	++	++	++	++	++	++	++	++	++
mannose	-	-	w	+	-	+	w	-	-	-
mannitol	-	-	-	-	-	-	-	-	-	-
melezitose	++	++	++	++	++	++	++	++	++	++
raffinose	++	++	++	++	++	++	++	++	++	++
ribose	-	+	++	+	++	++	++	++	++	++
sorbitol	-	-	-	-	-	-	-	-	-	-
sucrose	++	++	+	++	-	w	w	+	w	++
xylose	+	+	-	+	-	w	-	-	-	+

Symbol: ++ = strong reaction; + = positive reaction; w = weak reaction; - = negative reaction.

Table 2.3: continued

	Strains								
Carbohydrate	RC11	RC12	RC14	RC15	RC17	RC18	RC19	RC20	RC21
amygdalin	-	-	-	-	+	+	-	-	-
arabinose	-	-	-	-	w	-	-	-	-
esculin	-	-	-	-	-	-	++	-	-
glycogen	++	++	++	++	++	++	++	++	++
inositol	-	-	-	-	-	-	-	-	-
lactose	++	++	++	++	++	++	++	++	++
maltose	++	++	++	++	++	++	++	++	++
mannose	-	w	-	-	w	w	w	-	-
mannitol	-	-	-	-	-	-	-	-	-
melezitose	++	++	++	++	++	++	++	++	++
raffinose	++	++	++	++	++	++	++	++	++
ribose	++	+	++	++	++	++	++	++	++
sorbitol	-	-	-	-	-	-	-	-	-
sucrose	+	+	w	w	+	+	-	+	-
xylose	-	w	-	-	-	w	-	+	-

Symbol: ++ = strong reaction; + = positive reaction; w = weak reaction; - = negative reaction.

REFERENCES

1. Ashoor, S. H., and W. C. Monte. 1983. High performance liquid chromatographic determination of Bifidobacterium bifidum growth factors in human milk. J. Assoc. Off. Anal. Chem. 66: 135-139.
2. Azuma, N., K. Yamauchi, and T. Mitsuoka. 1984. Bifidus growth-promoting activity of a glycomacropeptide derived from human k-casein. Agric. Biol. Chem. 48: 2159-2162.
3. Beerens, H., C. Romond, and C. Neut. 1980. Influence of breast-feeding on the bifid flora of the newborn intestine. The Amer. J. Clin. Nutri. 33: 2434-2439.
4. Benno, Y. , K. Sawada, and T. Mitsuoka. 1984. The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. Microbiol. Immunol. 28: 975-986.
5. Benno, Y. , K. Suzuki, K. Suzuki, K. Narisawa, W. R. Bruce, and T. Mitsuoka. 1986. Comparison of the fecal microflora in rural Japanese and urban Canndians. Microbiol. Immunol. 30: 521-532
6. Bullen, C.L. ,and P. V. Tearle. 1976. Bifidobacteria in the intestinal tract of infants: an in-vitro study. J. Med. Microbiol. 9: 335-344.
7. Bullen, C.L., P.V. Tearle, and A.T.Willis. 1976. Bifidobacteria in the intestinal tract of infants: an in-vivo study. J. Med. Microbiol. 9: 325-333.
8. Hentges, D. J. 1983. Human intestinal microflora in health and disease. Academic press, Inc.

9. Minami,Y., K. Yazawa, Z. Tamura, T. Tanaka, and T. Yamamoto. 1983. Selectivity of utililization of galactosyl-oligosaccharides by bifidobacteria. Chem. Pharm. Bull. 31: 1688-1691.

10. Minami, Y., K. Yazawa, K. Nakamura, and Z. Tamura. 1985. Selectivity and efficiency of utilization of galactosyl-oligosaccharides by bifidobacteria. Chem. Pharm. Bull. 33: 710-714.

11. Mitsuoka, T., and C. Kaneuchi. 1977. Ecology of the bifidobacteria. The Amer. J. Clin. Nutri. 30: 1799-1810.

12. Larson, G., P. Falk, and L. C. Hoskins. 1988. Degradation of human intestinal glycosphingolipids by extracellular glycosidases from mucin-degrading bacteria of the human fecal flora. J. Biol. Chem. 263: 10790-10798.

13. Rasic, J. L., and J. A. Kurman. 1983. Bifidobacteria and their role. Library of congress cataloging in publication data.

14. Scardovi, V. 1981. The genus Bifidobacterium. in " The prokaryotes: a handbook on habitats, isolation, and identification of bacteria. vol.2 edited by M. P. Starr." p. 1951-1961.

15. Yoshioka, H., K. I. Iseki, and K. Fujita. 1983. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics. 72: 317-321.

CHAPTER 3

PLASMID PROFILES OF FECAL BIFIDOBACTERIA

ABSTRACT

The plasmid profiles of 35 strains of bifidobacteria from human sources were examined. Only one strain, B. breve 15698, harbored a 5.8 Kb (3.77 Md) plasmid. A curing process using the UV-light treatment to remove the plasmid was carried out, but characteristics of the cured strain were identical to those of the plasmid-containing strain, indicating the plasmid is cryptic.

INTRODUCTION

Plasmids are defined as autonomous replicating extrachromosomal genetic elements within the bacteria cell. These elements can be widely disseminated among the bacterial population by natural transfer mechanisms, such as transformation, transduction, or conjugation (3). Phenotypic and physical evidence for plasmid encoded properties in lactic acid bacteria have been obtained for citrate, sucrose, galactose, glucose, mannose, and xylose utilization, proteinase activity, modification/restriction system, as well as for drug resistance (7,10,11). Exclusive of their role as carriers and transporters of genetic information, plasmids can also provide a means of strain typing (15,16).

It is now well established that plasmids of diverse sizes are found within lactic acid bacteria (4,6,8), and plasmid biology is fast becoming an important area of research in these bacteria important in milk, meat and plant fermentation processes (4,6,8,11). Bifidobacteria are normal constituents of the intestinal flora of humans, and various animals (13,15). Most species are found almost exclusively in the intestinal tract of the host organism. Recently, reports have described the presence of plasmids in the genus Bifidobacterium (16,17,18), but only four species were represented: B. longum, the bifid species in the human intestine; B. globosum, the most common in animals; B. asteroides and B. indicum, species found exclusively in the intestine of honey bees. These reports indicate that from human sources, only B. longum harbors plasmids with sizes

ranging from 1.25 to 9.5 MDal. Recovery of different sizes of plasmids using different purification protocols have been observed (1). The purpose of the present study was to use published methods to isolate the plasmids of bifidobacteria isolated from humans.

MATERIALS AND METHODS

REAGENTS

The following materials were obtained from Sigma Chemical Co., St. Louis, MD: EDTA, sodium dodecyl sulfate, sodium hydroxide, Tris-hydroxide, sodium chloride, isoamyl alcohol, ribonuclease, ethidium bromide, magnesium sulfate, sucrose; the following were obtained from Difco laboratories, Detroit, MI: Reinforced Clostridial Medium (RCM), Reinforced Clostridial Agar (RCA), lysozyme; other materials were obtained from the companies indicated: Oxyrase enzyme system (Oxyrase Inc., Ashland, Ohio); phenol (International Biotechnologies, Inc., New Haven, CT); chloroform (J.T. Baker Inc., Phillipsburg, NJ); agarose (Bio-Rad Laboratories, Richmond, CA); supercoiled ladder DNA (Bethesda Research laboratories, Gaithersburg, MD); ethanol (Midwest Solvent Co., Pekin, IL).

BACTERIAL STRAINS

Bifidobacterium bifidum 15696, B. infantis 15697, B. breve 15698, B. breve 15700, B. infantis 15702, B. adolescentis 15703, B. longum 15707, and B. infantis 25962 were purchased from American Type Culture Collection, Rockville, MD. Bifidobacterium longum L1, L4, L10, L12, and B. bifidum L6 were isolated from the feces of 15-day-old breast-fed baby L; B. breve T2, T7, T9, and T11, were isolated from the feces of 15-day-old breast-fed baby T; and B. spp RC1 ,RC2, RC3, RC4, RC5, RC6, RC7, RC8, RC9, RC10, RC11, RC12, RC14,

RC15, RC17, and RC18, were isolated from the feces of 3-month-old breast-fed baby RC (for fecal bifidobacteria characterization, see Chapter two). Bifidobacterium longum 1j, and 3j were isolated from a commercial preparation of " bifidus powder" (Calpis Food Industry Co., LTD, Japan). All strains were maintained by weekly transfer in RCM and incubated anaerobically at 37°C for 12 hours.

Escherichia coli V517 containing 8 plasmids ranging in size from 1.4 to 35.8 megadaltons (MDal) served as the mobility reference standard during agarose gel electrophoresis (9).

GROWTH OF CELLS AND DNA PREPARATION

Strains used for plasmid DNA extration were grown in RCM at 37°C for 12 hours under anaerobic conditions. The resulting culture was used to provide a 0.05% inoculum in RCM containing 0.15 unit/ml oxyrase; this was the lysis broth. Strains were propagated overnight at 37°C and harvested by centrifugation at 6000 rpm for 15 min.

ISOLATION OF PLASMID DNA

Plasmid DNA was isolated according to the method described by Anderson and McKay (1) with slight modification. Cell pellets were resuspended in 379 ul of sucrose buffer. This suspension was vortexed and treated with 96.5 ul lysozyme (10 mg/ml), followed by incubation at 37°C for 30 min. After addition of 48.2 ul of 0.25 M EDTA, lysis was effected by the addition of 27.6 ul of 20% sodium

dodecyl sulfate with gentle mixing. DNA was then sheared by vortexing at high speed for 30 sec. Fresh 3.0 N sodium hydroxide (27.6 ul) was added and mixed gently by intermittent inversion or swirling. Subsequently, the lysate was treated with 49.6 ul of 2.0 M Tris-hydrochloride (pH 7.0) and 71.7 ul of 5.0 M sodium chloride and vortex gently but thoroughly. Phenol saturated with 3% sodium chloride (0.7 ml) was added and vortexed at high speed for 5 sec followed by centrifugation at 13,000 rpm for 5 min at room temperature. The upper phase was removed and extracted with 0.7 ml chloroform: isoamyl alcohol (24:1) and then centrifugated at 13,000 rpm for 1 min. The upper phase was removed and the DNA therein precipitated by addition of 1 ml of 95% ethanol and incubation at 0°C for at least 30 min. After incubation, the ethanol precipitated DNA was centrifuged at 13,000 rpm for 15 min and the supernatant poured off. Pellets were dried and resuspended in 15 ul of 10mM Tris- 1mM EDTA (pH 8.0) buffer and treated with 2 ul of 1 mg/ml ribonuclease solution and 3 ul of loading buffer (25% Ficoll, 0.1% bromophenol blue, 0.1% SDS in TE pH 8.0). The DNA sample was analyzed immediately by gel electrophoresis.

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed in a Tris acetate buffer containing 40 mM Tris, 20 mM acetic acid, and 2mM EDTA (pH 8.0). The gel contained 0.7% agarose and electrophoresis was performed at 80 V for 90 min (EC-420, E-C Apparatus Corporation, St Petersburg, FL). After electrophoresis, the gel was stained by

immersion in 1 ug/ml of ethidium bromide solution for 15 min and destained in 1 mM magnesium sulfate for 10 min. Gels were photographed with a stationary Fotodyne DS34 camera equipped with a red filter using Polaroid-type 667 film.

CURING OF PLASMID DNA WITH UV LIGHT

Strain 15698 was grown in RCM at 37°C for 12 hours (GasPak, BBL system). Cells were spun down at 6000 rpm for 10 min and the pellets resuspended in 10 ml phosphate buffered saline (pH 6.5) containing 0.3 unit/ml oxyrase. The cell suspension cultures was placed in a 16 cm plate (Tek Tartor V shaker) on the platform of a rotatory shake(40rpm) and held for two hours. During this time cells were illuminated under UV light (GE 15 watt bulb) at a distance of 15 cm. Samples were taken at 0, 0.5, 1, 1.5, and 2-hour intervals, appropriately diluted in PBS solution and pour-plated into RCA incubated anaerobically for 36 hours (GasPak, BBL). Colonies were needle inoculated into RCM and plasmid profiles run according to the above mentioned method.

RESULTS

The methods described here for isolation of plasmid DNA from bifidobacteria were essentially according to a procedure previously published (1), but for Lactococcus bacteria. Surprisingly, the procedure worked well for Bifidobacterium. The quantity of cells was important for the success of the procedure. However, an excess of cells resulted in poor lysis because of insufficient digestion of the cell walls by lysozyme over the short incubation period. Too few cells was also undersirable because of the recovery of inadequate amounts of plasmid DNA. Under this condition, 0.05% or smaller inoculum permitted combining the convenience of overnight incubation with obtaining cultures in the late exponential phase of growth, which are more amenable to lysis than stationary phase cells. A group of 35 strains belonging to the 5 species (B. longum, B. infantis, B. breve , B. bifidum, and B. adolescentis), and with some human isolates not identified to species were examined for plasmid profiles. Profiles from representative strains are shown in Figures 3.1 to 3.2. All strains had no plasmids, except for B. breve 15698, which harbored a single 5.8 Kb (3.77 MDal) plasmid. These results confirmed previous reports that plasmids are rare in the genus Bifidobacterium among isolates from human sources. The plasmid of B. breve was cured by UV-light illumination and the mutant strain 15698-UV10 was compared to the wild type for antibiotic resistance and sugars fermented as shown in Table 3.1. No differences were

found between the parent and cured strains; consequently no function could be attributed to the plasmid and it remains cryptic.

DISCUSSION

Bifidobacteria constitute one of the dominant bacterial groups of the human intestine. Since Mechnikoff's time, several lactobacilli, including L. acidophilus and L. bifidus (later called Bifidobacterium bifidum), have been investigated as possible candidate organisms for dietary adjuncts (5). Recent progress in the genetics of lactic acid bacteria, particularly those of the Lactococcus, Lactobacillus , Leuconostoc , and Pediococcus genera have established the essential involvement of plasmid DNA in numberous activities related to fermentation performance (4,6,7,8,10,11,14). Unfortunately, plasmid genetics of the Bifidobacterium has not kept pace with advances in molecular genetics for other lactic acid type bacteria. Clearly, the study of plasmid biology in dairy and food starter cultures has become a prerequisite for future strain improvement programs. This knowledge, coupled with developing plasmid transfer systems for lactic acid bacteria, is essential for future gene cloning work within this group of organism. Because of their anaerobic characteristics, Bifidobacterium species have not received much attention in genetics research. In 1982, Sgorbati et al. (16) first isolated plasmids from bifidobacteria and from their survey of human bifid species, only B. longum harbored plasmid, whereas B. infantis and B. breve did not. In our survey of 35 strains of bifidobacteria, only strain B. breve 15698 harbored a plasmid. This result was contrary to the findings of Sgorbati et al. (16). In their study, only B. longum harbored plasmids and 70% of these strains had plasmids with thirteen different profiles consisting of two to seven plasmid bands with a

size range of 1.25 to 9.5 MDal. The moleclar weight (MW) of the plasmid band isolated from B. breve 15698 was estimated by preparing a standard curve as described by Meyers (12) based on the gel migration distances of supercoiled ladder DNA. Plasmid DNA in the closed circle configuration will migrate through an agarose gel of this concentration at a rate inversely related to its MW. The MW of uncharacterized bands could then be estimated by reading their migration distances against the standard curve. Strain 15698 in our survey was found to harbor a plasmid of 5.8 Kb (Fig. 3.4). Further characterization of this strain showed it was fructose-6-phosphate phosphoketolase (F6PPK) positive and that it fermented eight sugars (Table 3.1). Strain 15698 was similar to B. breve ss. parvulorum (13), but melezitose and amygdalin were not fermented compared to variable and positive results, respectively and glycogen was fermented compared to variable result in Mitsuoka et al. (13). However, B. longum, B. breve, and B. infantis are closely related: DNA homology between B. longum and B. infantis is 65 to 80%, and B. breve shows 50 to 75% homology with both (16).

In order to determine whether or not the 5.8Kb (3.77 Md) plasmid was associated with any biological function, plasmid curing was first attempted with acriflavin, but B. breve 15698 was very sensitive to this compound as no growth was found at acriflavin levels higher than 1.2 micrograms per ml. Likely, because of this sensitivity, the plasmid was not cured. Gradually, B. breve 15698 was induced to grow a higher concentration of acriflavin (50 microgram/ml), but curing did not occur at this level either (Fig. 3.3). Reasons why the acriflavin did not cure the plasmid is not

understood, since it has been used to cure the plasmids in many other bacteria (7). Another curing agent, UV-light, was therefore used; after 2 hours of illumination, the death rate was 99.9999% (5 x 10^8 CFU/ml to 3 x 10^2 CFU/ml) and randomly selected colonies were analyzed for the plasmid profiles. One isolate was found (Fig. 3.3) with the plasmid band missing. The UV-light may have caused thymidine dimer formation in DNA, but the exact mechanism of curing is still unsolved. The mutant strain, 15698-UV10, and its parent, 15698, were compared for sugar fermentation ability as well antibiotic resistance. No differences were found (Table 3.1). More research therefore is necessary to determine whether or not this plasmid encodes for any function useful to the cell.

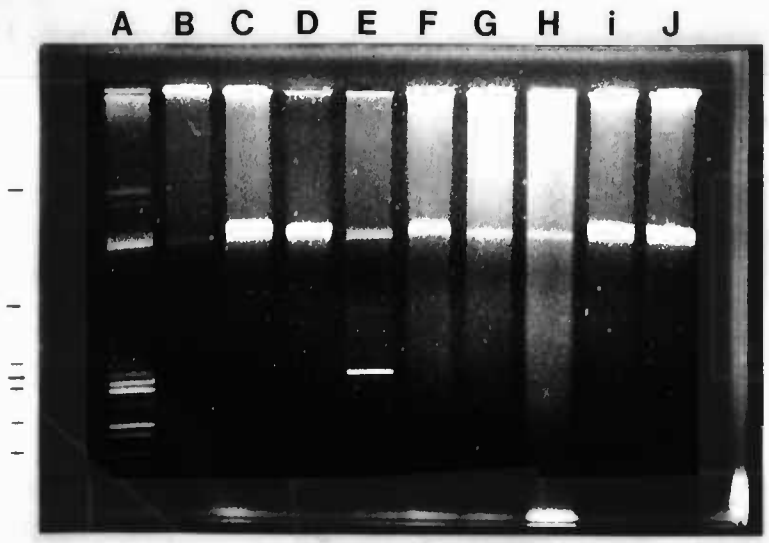

Figure. 3.1. Plasmid profiles of bifidobacteria. Lane A, E. coli V517 reference plasmids with the following molecular weights in Megadaltons (Md.): top to bottom, 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, and 1.4. Lane B, B. bifidum 15696; lane C, B. breve 15700; lane D, B. longum 15707; lane E, B. breve 15698; lane F, B. longum L12; lane G, B. breve T2; lane H, B. breve T11; lane I, B. longum 1J; lane J, B. longum 3J.

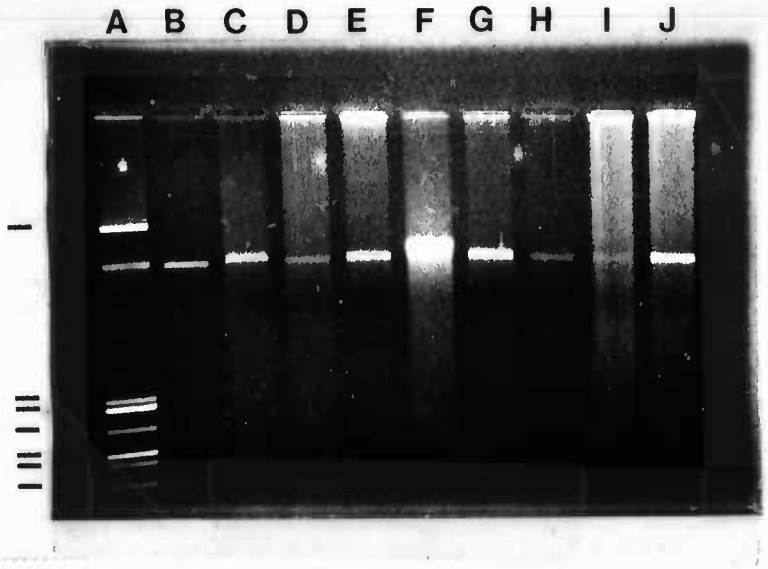

Figure. 3.2. Plasmid profiles of bifidobacteria. Lane A, E. coli V517 reference plasmids with the following molecular weights in Megadaltons (Md.): top to bottom, 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, and 1.4. Lane B, B. longum L1; lane C, B. longum L4; lane D, B. breve T7; lane E, B. breve T9; lane F, B. spp. RC1; lane G, B. spp. RC3; lane H, B. spp. RC12; lane I, B. spp. RC 15; lane J, B. spp. RC 18.

Figure. 3.3. Plasmid profiles of B. breve 15698 after treatment with two different curing agents acriflavin (ug/ml) and UV-light (2 hours at 15cm distance). Lane A, E. coli V517 reference plasmids with the following molecular weights in Megadaltons(Md.): top to bottom , 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, and 1.4; lane B to lane E with UV-light treatment; lane F to I with acriflavin treatment; lane J, control B. breve 15698.

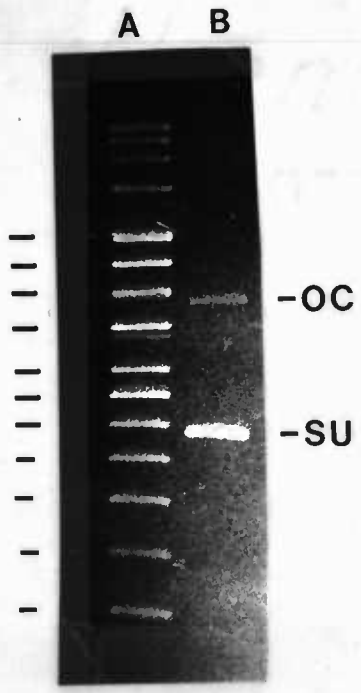

Figure. 3.4. Estimation of size of plasmid DNA of B. breve 15698 from large scale DNA preparation. Lane A, ladder DNA of the following kilobase (Kb) size: top to bottom, 16.210, 14.174, 12.138, 10.102, 8.066, 7.048, 6.030, 5.012, 3.990, 2.972, and 2.067. Lane B, B. breve 15698. OC: open circle DNA. SU: supercoil DNA.

Table 3.1: Comparison of properties of plasmid cured strain 15698-UV10 with its parent strain 15698.

	Strains	
	15698	15698-UV10
Antibiotics:		
nalidixic acid	R	R
kanamycin sulfate	R	R
polymyxin B sulfate	R	R
Carbohydrate:		
amygdalin	-	-
arabinose	-	-
esculin	+	+
glycogen	+	+
inositol	-	-
lactose	++	++
maltose	++	++
mannose	+	+
mannitol	-	-
melezitose	-	-
raffinose	++	++
ribose	+	+
sorbitol	-	-
sucrose	++	++
xylose	-	-

Table 3.1: continued

Symbols: R = resistant; ++ = strong positive; + = positive
w = weak positive; - = negative.

REFERENCES

1. Anderson, D. G., and L. L. McKay. 1983. Simple and rapid method for isolation large plasmid DNA from lactic Streptococci. App. Enviro. Microbiol. 46: 549-552.
2. Brigidi, P., D. Matteuzzi, and F. Crociani. 1986. Protoplast formation and regeneration in Bifidobacterium. Microbiologica 9: 243-248.
3. Broda, P. 1979. Plasmid. Oxford and San francisco: W. H. Freeman.
4. Davies, F. L., H. M. Underwood, and M. J. Gasson. 1981. The value of plasmid profiles for strain identification in lactic Streptococci and the relationship between Streptococcus lactis 712, ML3 and C2. J. Appl. Bacteriol. 51: 325-337.
5. Kim, H.S.. 1988. Characterization of lactobacilli and bifidobacteria as applied to dietary adjuncts. Cult. Dairy Prod. J. p6-9
6. Klaenhammer, T. R., L. L. McKay, and K. A. Baldwin. 1978. Improved lysis of group N streptococci for isolation and rapid characterization of plasmid deoxyribonucleic acid. App. Environ. Microbiol. 35: 592-600.
7. Kondo, J. K., and L. L. McKay. 1985. Gene transfer systems and molecular cloning in group N Streptococci: a review. J. Dairy Sci. 68: 2143-2159.
8. Leblanc, D. J., and L. N. Lee. 1979. Rapid screening procedure for detection of plasmids in Streptococci. J. Bacteriol. 140: 1112-1115.

9. Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayres, S. M. McCowen. 1978. A multiple plasmid-containing Escherichia coli strain: convenient source of size reference plasmid molecules. Plasmid 1: 417-420.

10. McKay, L.L.. 1983. Functional properties of plasmids in lactic Streptococci. Antonie van Leeuwenhoek. 49: 259-274.

11. McKay, L. L. 1985. Roles of plasmids in starter cultures. in " Bacterial starter cultures for foods. edited by S. E. Gilliland" CRC press, Inc.

12. Meyers, J. A., D. Sanchez, L.P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127: 1529-1537.

13. Mitsuoka, T., and C. Kaneuchi. 1977. Ecology of the bifidobacteria. Amer. J. Clin. Nutri. 30: 1799-1810.

14. Orberg, P. K., and W. E. Sandine. 1984. Microscale method for rapid isolation of covalently closed circular plasmid DNA from group N Streptococci. App. Enviro. Microbiol. 47: 677-680.

15. Scardovi, V., F. Casalicchio, N. Vincenzi. 1979. Multiple electrophoretic forms of transaldolase and 6-phosphogluconate dehydrogenase and their relationship to the taxonomy and ecology of bifidobacteria. Intern. J. System. Bacteriol. 29: 312-329.

16. Sgorbati, B., V. Scardovi, and D. J. Leblanc. 1982. Plasmids in the genus Bifidobacterium. J. General. Microbiol. 128: 2121-2131.

17. Sgorbati, B., V. Scardovi, and D. J. Leblanc. 1986. Related structures in the plasmid profiles of Bifidobacterium longum. Microbiologica. 9: 415-422.

18. Sgorbati, B., V. Scardovi, and D. J. Leblanc. 1986. Related structures in the plasmid profiles of Bifidobacterium asteroides, B. indicum and B. globosum. Microbiologica 9: 443-456.

19. Walsh, P. M., and L. L. McKay. 1981. Recombinant plasmid associated with cell aggregation and high-frequency conjugation of Streptococcus lactis ML3. J. Bacteriol. 146: 937-944.

20. Yu, R. S., T. V. Hung, and A. A. Azad. 1982. Rapid screening of highly purified plasmids in lactic Streptococci. Aust. J. Dairy Technol. 37: 99- 103.

BIBLIOGRAPHY

1. Adler, H. I., and W. D. Crow. 1981. A novel approach to the anaerobic microorganisms. Biotechnology and Bioengineering Symp. No. 11, 533-540.
2. Adler, H. I., W. D. Crow, C. T. Hadden, J. Hall, and R. Machanoff. 1983. New techniques for growth anaerobic bacteria: Experiments with Clostridium butyricum and Clostridium acetobutylicum. Biotechnology and Bioengineering Symp. No 13, 153-161.
3. Anderson, D. G., and L. L. McKay. 1983. Simple and rapid method for isolation large plasmid DNA from lactic Streptococci. App. Enviro. Microbiol. 46: 549-552.
4. Ashoor, S. H., and W. C. Monte. 1983. High performance liquid chromatographic determination of Bifidobacterium bifidum growth factors in human milk. J. Assoc. Off. Anal. Chem. 66: 135-139.
5. Azuma, N., K. Yamauchi, and T. Mitsuoka. 1984. Bifidus growth-promoting activity of a glycomacropeptide derived from human k-casein. Agric. Biol. Chem. 48: 2159-2162.
6. Beerens, H., C. Romond, and C. Neut. 1980. Influence of breast-feeding on the bifid flora of the newborn intestine. The Amer. J. Clin. Nutri. 33: 2434-2439.
7. Benno, Y. , K. Sawada, and T. Mitsuoka. 1984. The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. Microbiol. Immunol. 28: 975-986.

8. Benno, Y. , K. Suzuki, K. Suzuki, K. Narisawa, W. R. Bruce, and T. Mitsuoka. 1986. Comparison of the fecal microflora in rural Japanese and urban Canndians. Microbiol. Immunol. 30: 521-532

9. Brigidi, P., D. Matteuzzi, and F. Crociani. 1986. Protoplast formation and regeneration in Bifidobacterium. Microbiologica 9: 243-248.

10. Broda, P. 1979. Plasmid. Oxford and San francisco: W. H. Freeman.

11. Bullen, C.L. ,and P. V. Tearle. 1976. Bifidobacteria in the intestinal tract of infants: an in-vitro study. J. Med. Microbiol. 9: 335-344.

12. Bullen, C.L., P.V. Tearle, and A.T.Willis. 1976. Bifidobacteria in the intestinal tract of infants: an in-vivo study. J. Med. Microbiol. 9: 325-333.

13. Carlsson, J., G. Nyberg, and J. Wrethen. 1978. Hydrogen peroxide and superoxide radical formation in anaerobic broth media exposed to atmospheric oxygen. App. Enviro. Microbiol. 36: 223-229.

14. Carlsson, J., G. P. D. Granberg, G. K. Nyberg, and M. K. Edlund. 1979. Bactericidal effect of cysteine exposed to atmospheric oxygen. App. Enviro. Microbiol. 37: 383-390.

15. Collins, E. B., and B. J. Hall. 1984. Growth of bifidobacteria in milk and preparation of Bifidobacterium infantis for a dietary adjunct. J. Dairy Sci. 67: 1376-1380.

16. Crow, W. D., R. Machanoff, and H. I. Alder. 1985. Isolation of

anaerobes using an oxygen reducing membrane fraction: experiments with acetone butanol producing organisms. J. Microbiol. Methods. 4:133-139.

17. Davies, F. L., H. M. Underwood, and M. J. Gasson. 1981. The value of plasmid profiles for strain identification in lactic Streptococci and the relationship between Streptococcus lactis 712, ML3 and C2. J. Appl. Bacteriol. 51: 325-337.

18. Deguchi, Y., T. Morishita, and M. Mutai. 1985. Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. Agri. Biol. Chem. 49: 13-19.

19. Faure, J. C., D. Schellenberg, A. Bexter, and H. P. Wurzner. 1982. Barrier effect of Bifidobacterium on Escherichia coli in the germ-free rat. Int. J. Vitam. Nutr. Res. 52:225-230.

20. Ferrari, A. , N. Pacini, and E. Canzi. 1980. A note on bile acids transformations by strains of Bifidobacterium. J. Appl. Bacteriol. 49: 193-197.

21. Gilliland, S. E. 1977. The manufacture of starters by batch fermentation and centrifugation to produce concentrates. J. Soc. Dairy Technol. 30: 36-41.

22. Gilliland, S. E., M. L. Speck, G. F. Nauyok, and F. G. Giesbrecht. 1978. Influence of consuming nonfermented milk containing Lactobacillus acidophilus on fecal flora of healthy males. J. Dairy Sci. 61: 1-10.

23. Hentges, D. J. 1983. Human intestinal microflora in health and disease. Academic press, Inc.

24. Hoskins, C. B., E. Rico-Munoz, and P. M. Davidson. 1986. Aerobic incubation of Clostridium perfringens in the presence

of an oxygen-reducing membrane fraction. J. Food Sci. 51: 1585-1586.

25. Kim, S. H. 1988. Characterization of Lactobacilli and Bifidobacterium as applied to dietary adjunts. Cult. Dairy Prod. J. 6-9.

26. Klaenhammer, T. R., L. L. McKay, and K. A. Baldwin. 1978. Improved lysis of group N streptococci for isolation and rapid characterization of plasmid deoxyribonucleic acid. App. Environ. Microbiol. 35: 592-600.

27. Kondo, J. K., and L. L. McKay. 1985. Gene transfer systems and molecular cloning in group N Streptococci: a review. J. Dairy Sci. 68: 2143-2159.

28. Krieg, N. R., and P. S. Hoffman. 1986. Microaerophily and oxygen toxicity. Ann. Rev. Microbiol. 40: 107-130.

29. Lamprech, E. D., and E. M. Foster. 1963. The survival of starter organisms in concentrated suspensions. J. Appl. Bacteriol. 26: 359- 363.

30. Larson, G., P. Falk, and L. C. Hoskins. 1988. Degradation of human intestinal glycosphingolipids by extracellular glycosidases from mucin-degrading bacteria of the human fecal flora. J. Biol. Chem. 263: 10790-10798.

31. Leblanc, D. J., and L. N. Lee. 1979. Rapid screening procedure for detection of plasmids in Streptococci. J. Bacteriol. 140: 1112-1115.

32. Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayres, S. M. McCowen. 1978. A multiple plasmid-containing Escherichia coli

strain: convenient source of size reference plasmid molecules. Plasmid 1: 417-420.

33. McKay, L.L.. 1983. Functional properties of plasmids in lactic Streptococci. Antonie van Leeuwenhoek. 49: 259-274.

34. McKay, L. L. 1985. Roles of plasmids in starter cultures. in " Bacterial starter cultures for foods. edited by S. E. Gilliland" CRC press, Inc.

35. Meyers, J. A., D. Sanchez, L.P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127: 1529-1537.

36. Metchnikoff, E. 1908. The prolongation of life. 1st edition. G. P. Putnams sons, New York.

37. Minami,Y., K. Yazawa, Z. Tamura, T. Tanaka, and T. Yamamoto. 1983. Selectivity of utililization of galactosyl-oligosaccharides by bifidobacteria. Chem. Pharm. Bull. 31: 1688-1691.

38. Minami, Y., K. Yazawa, K. Nakamura, and Z. Tamura. 1985. Selectivity and efficiency of utilization of galactosyl-oligosaccharides by bifidobacteria. Chem. Pharm. Bull. 33: 710-714.

39. Mitsuoka, T., and C. Kaneuchi. 1977. Ecology of the bifidobacteria. The Amer. J. Clin. Nutri. 30: 1799-1810.

40. Nagawa, M., A. Nakabayyashi, and S. Fujino. 1988. Preparation of the bifidus milk powder. J. Dairy Sci. 71: 1777-1782.

41. Orberg, P. K., and W. E. Sandine. 1984. Microscale method for rapid isolation of covalently closed circular plasmid DNA from group N Streptococci. App. Enviro. Microbiol. 47: 677-680.

42. Patel, G. B., L. A. Roth, and B. J. Agnew. 1984. Death rates of obligate anaerobes exposed to oxygen and the effect of media prereduction on cell viability. Can. J. Microbiol. 30: 228-235.

43. Poch, M., and A. Bezkorovainy. 1988. Growth-enhancing supplements for various species of the Bifidobacterium. J. Dairy Sci. 71: 3214-3221

44. Poupard, J. A., L. Husain, and R. F. Norris. 1973. Biology of the bifidobacteria. Bacteriol. Rev. 37: 136-165.

45. Rasic, J. L., and J. A. Kurman. 1983. Bifidobacteria and their role. Library of congress cataloging in publication data.

46. Rolfe, R. D., D. J. Hentges, B. J. Campbell, and J. T. Barret. 1978. Factors related to the oxygen tolerence of anaerobic bacteria. App. Enviro. Microbiol. 36: 306-313.

47. Scardovi, V. 1981. The genus Bifidobacterium. in " The prokaryotes: a handbook on habitats, isolation, and identification of bacteria. vol.2 edited by M. P. Starr." p. 1951-1961.

48. Scardovi, V., F. Casalicchio, N. Vincenzi. 1979. Multiple electrophoretic forms of transaldolase and 6-phosphogluconate dehydrogenase and their relationship to the taxonomy and ecology of bifidobacteria. Intern. J. System. Bacteriol. 29: 312-329.

49. Sgorbati, B., V. Scardovi, and D. J. Leblanc. 1982. Plasmids in the genus Bifidobacterium. J. General. Microbiol. 128: 2121-2131.

50. Sgorbati, B., V. Scardovi, and D. J. Leblanc. 1986. Related structures in the plasmid profiles of Bifidobacterium longum. Microbiologica. 9: 415-422.

51. Sgorbati, B., V. Scardovi, and D. J. Leblanc. 1986. Related structures in the plasmid profiles of Bifidobacterium asteroides, B. indicum and B. globosum. Microbiologica 9: 443-456.

52. Staab, J. A., and J. K. Ely. 1987. Viability of lyophilized anaerobes in two media. Cryobiology 24: 174-178.

53. Stark, P. L., and A. Lee. 1982. The microbial ecology of large bowel of breast-fed and formula-fed infants during the first year. J. Med. Microbiol. 15: 189-203.

54. Sykes, G., and F. A. Skinner. 1983. Techniques for the isolation and characterization of Actinomyces and Bifidobacterium species: report of a panel discussion. Soc. Appl. Bact. Symp. Series No.2 p. 327-333.

55. Walsh, P. M., and L. L. McKay. 1981. Recombinant plasmid associated with cell aggregation and high-frequency conjugation of Streptococcus lactis ML3. J. Bacteriol. 146: 937-944.

56. Willrett, D. L., W. E. Sandine, and J. W. Ayres. 1979. Evaluation of a new pH-controlled bulk starter medium. Cheese reporter 103: 8-9

57. Wright, S. L., and G. H. Richardson. 1982. Optimization of whey-based or nonfat dry milk-based media for production of pH controlled bulk lactic cultures. J. Dairy Sci. 65: 1882-1889.

58. Yoshioka, H. Y., K.Iseki, and K. Fujita. 1983. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics 72: 317-321.

59. Yu, R. S., T. V. Hung, and A. A. Azad. 1982. Rapid screening of highly purified plasmids in lactic Streptococci. Aust. J. Dairy Technol. 37: 99- 103.