CLINICAL AND BIOCHEMICAL STUDIES ON THE GROWTH OF LACTOBACILLUS BIFIDUS

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It has been established that Lactobacillus bifidus is the predominant intestinal flora in breast fed infants, however, its significance in infant's nutrition seems to be a subject of controversy. In our laboratory, a number of studies have been undertaken on the basis of the following assumptions;

1. Human milk is of first choice for infant nutrition and an ultimate goal for the improvement of the bottle feeding is to modify cow's milk toward human milk mainly from the qualitative viewpoint.
2. Such efforts can be reflected on the intestinal flora of infants, especially on the bifidus number as an indicator for the modification of milk, and also to pursue the condition suitable for the multiplication of L. bifidus would throw light upon clarifying the biological differences between human and cow's milk.

It is generally accepted that L. bifidus shows a satisfactory growth on the Petuely's media and Plotho's media under the anaerobic condition. Recently, Tamaki[1] in our laboratory succeeded in culturing L. bifidus aerobically in the semisynthetic media (K-media; devised in our laboratory), to which pyruvic acid was added. Moreover, in place of pyruvic acid, the addition of substance chosen from organic acids, amino acids, fatty acids, purines, nucleic acids, vitamines or glucosamine derivatives resulted in a satisfactory growth of L. bifidus.

In the meantime, Kuniya[2] in our laboratory studied on the significance of carbohydrates of milk for the growth of L. bifidus and it was confirmed that of all the sugars tested, lactose was the most active for the growth of L. bifidus, either in vitro or in vivo, and that lactose was a kind of special sugar known as structural sugar, which would be significant for the bifidus growth from the aspect not only of the source of energy but also of the structure itself. The idea of my present study was suggested by those two investigations and dealt with the influences of the test material on the growth of intestinal flora when each test material was injected directly into the ileocecum of infants, using the Kobe-Tubing procedure[3] which was devised in our department. A main result of this experiment was that the same predominant bifidus flora just as seen in breast fed infants, was inducible by the administration of test materials. The mechanism of

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this result will be discussed mainly from the biochemical aspect.

Part I. Growth of L. Bifidus upon Injection of Test Material with Lactose into the Ileocecum of Infants

Experimental Method

a. *Examinees*

Healthy infants, aged from 2 to 6 months, who were fed with the basic formula (Mixture of 13 percent evaporated milk, 5 percent dextrimaltose and 2 percent Biocemeal). Their stool before study were of the typical mixed flora.

b. *Test Material*

Referring to Tamaki's[1] in vitro study, the main compounds as indicated below, were chosen from the group whose microbiological activity was compared with that of pyruvic acid.

1. Organic acids; α–ketoglutaric acid, citric acid, lactic acid, acetic acid, oxaloacetic acid, succinic acid and fumaric acid.
2. Amino acids: Aspartic acid, glutamic acid, alanine, glycine and lysine.
3. Fatty acid: Palmitic acid, propionic acid, butyric acid and linoleic acid.
4. Purine derivatives: guanine and uracil.
5. Vitamines: Vitamine B_1, Thiamine pyrophosphate (TPP), FAD (flavin adenine dinucleotide) and vitamine C.
6. Glucosamine derivatives: D–glucosamine, N–acetyl–D–glucosamine and β–ethyl–N–acetyl–D–glucosamine.

c. *Method of Injection of Test Materials into the Ileocecum of Infants.*

10 cc. of the solution containing 0.5 percent single test material and 20 percent lactose were injected every 4 hours into the intestinal lumen of the ileocecum of infants, fed with the basic formula, using the Kobe-Tubing procedure. Observations were continued for 30 hours after administration.

d. *Method of Observation.*

1. Stool smears: Stool specimens were obtained with a special collection tube, immediately before each test material was injected every 4 hours. Each specimen was diluted with 10 folds of normal saline. Smears were stained by modified Gram's method (Hucker's method) and examined microscopically.
2. Cultures: At the period of the maximum growth of L. bifidus on smears, a small fecal mass was taken with a collection tube from the anus of breast fed infants. The specimens were emulsified with normal saline to the extent that the number of colonies were counted about 100. The inoculum was then cultured on the K-broth[1] by the streaked-plate method under the anaerobic condition (using white phosphorus), at 37°C for 72 hours. The colonies were stained by the modified Gram's method (Hucker's me-

thod) and the percentage of the population of L. bifidus were obtained.

Table 1. K-broth; devised semisynthetic medium for L. bifidus.

Pyruvic acid	1.0 ml.
Lactose	3.0 gm.
Peptone	0.5 gm.
Casein hydrolysate	1.0 ml.
Yeast extract	0.05 gm.
Cystine	0.02 gm.
*Salt mixture	0.5 ml.
Distilled water added to	100.0 ml.

*Salt mixture

$MgSO_4 7H_2O$	1.0 gm.
$FeSO_4 7H_2O$	0.5 gm.
NaCl	0.5 gm.
$MnSO_4 2H_2O$	0.337gm.
Distilled water added to	250.0 ml.

3. Other findings: Frequency, character and PH of stools, general condition of infants, body weight gain, daily milk intake and body temperature.

Results

A. *Injection of Organic Acid with Lactose into Ileocecum of Infants.*

Table 2. Smears and cultures for L. bifidus upon injection of organic acid with lactose into ileocecum of infants.

	Smears			48 hrs. after discontinued injection	Character of stool	Cultures Bifidus %
	8–12 hrs.	16 hrs.	24 hrs.			
α-Ketoglutaric acid	Gram-positive cocci and negative rods decreased, and Gram-positive rods (L. bifidus) increased.	Pure bifidus flora	Pure bifidus flora	Returned to the same mixed flora as before injection	Yellow, soft and acid	90.3%
Oxaloacetic A.						84.6%
Citric A.	Bifidus increased	Gram-positive cocci increased gradually	Remained same	Returned to the mixed flora	Light yellow, soft and acid	20.7%
Lactic A.	Mixed flora	Mixed flora	Bifidus slightly increased	Returned to the mixed flora	Light yellow, soft, and slightly alkaline	31.2%
Succinic A.	Almost pure bifidus flora	Bifidus slightly decreased	Bifidus re-increased	Remained same	Yellow, soft and slightly acid or alkaline	78.0%
Fumaric A.	Bifidus increased.	Bifidus increased	Almost pure bifidus flora	Remained same	Yellow, very soft and acid	69.0%

Table 2 shows that L. bifidus has mostly increased 12 to 16 hours after the first administration of each organic acid tested, combined with lactose into the ileocecum. α-ketoglutaric acid, oxaloacetic acid and succinic acid were found to be markedly active to the extent that the pure bifidus flora was obtained. In the meantime, the character of stool also has become similar to that of breast fed infants.

Other findings

No change in frequency of stool could be noted, but stools tended to be soft. General condition of infants remained excellent and amount of milk intake was unchanged. Weight gain seemed to be satisfactory and any side effects such as fever, vomiting or allergy were not observed.

B. *Injection of Amino Acid with Lactose into Ileocecum of infants.*

Table 3. Smears and cultures for L. bifidus upon injection of amino acid with lactose into ileocecum of infants.

	Smears				Character of stool	Cultures Bifidus %
	8-12 hrs.	16 hrs.	24 hrs.	48 hrs. after discontinuing injection		
Aspartic acid	—	Pure bifidus flora	Pure bifidus flora	Returned to mixed flora	Soft pH: 5.5~6.6	84.5%
Glutamic acid	Bifidus increased (60%)	Remained same	Remained same	Returned to mixed flora	Soft pH: 5.6~6.6	72.4%
Lysine	Bifidus increased (70%)	Remained same	Bifidus over 70%	Returned to mixed flora	Soft pH: 5.6~6.2	78.8%
Alanine	—	Bifidus(30%) Many Gram Positive cocci	—	Returned to mixed flora	Soft pH: 6.0	41.3%
Glycine	—	Pure bifidus flora	Pure bifidus flora	Returned to mixed flora	Soft pH: 6.0	62.3%

As shown in Table 3, aspartic acid, glutamic acid and lysine showed a considerable growth promoting activity for L. bifidus in vivo.

C. *Injection of Fatty Acid with Lactose into Ileocecum of Infants.*

Table 4. Smears and cultures for L. bifidus upon injection of fatty acid with lactose into ileocecum of infants.

	Smears				Character of stools	Cultures Bifidus %
	8-12 hrs.	16 hrs.	24 hrs.	24-48 hrs. after discontinuing injection		
Propionic acid	Bifidus increased (50%)	Remained same	Bifidus decreased	Returned to mixed flora	pH: 5.2~6.4	51.8%

Butylic acid	Gram-positive cocci (60%)	Bifidus(20%)	Bifidus(30%)	Mixed flora	pH: 5.0~5.4	24.5%
Palmitic acid	Gram-positive cocci (80%)	Gram-positive cocci over 80%	Remained same	Mixed flora	—	8.1%
Linoleic acid	Gram-positive cocci over 80%	Remained same	Remained same	Mixed flora	—	8.1%

It should be noted that there was remarkable increase of growth of the Gram positive cocci in response to the administration of fatty acid, whereas L. bifidus was unaffected.

D. *Injection of Purine Derivatives with Lactose into Ileocecum of Infants.*

Among various kinds of purine derivatives, guanine and uracil, which were proved active for L. bifidus in vitro, were used for this experiment.

Table 5. Smears and cultures for L. bifidus upon injection of purine derivative with lactose into ileocecum of infants.

	Smears				Character of stool	Culture Bifidus %
	4 hrs.	8–12 hrs.	16 hrs.	24 hrs.		
Guanine	Bifidus increased	Bifidus 40%	Remained same	Remained same	Soft pH: 6.0	13.8%
Uracil	—	Bifidus markedly increased	—	—	Soft pH: 6.0	77.2%

As seen in Table 5, a fairly accelerated growth promoting activity for L. bifidus was observed with uracil, but not with guanine.

E. *Injection of Vitamines with Lactose into Ilececum of Infants.*

Table 6. Smears and cultures for L. bifidus upon injection of vitamine with lactose into ileocecum of infants.

	Smears		Character of stool	Culture Bifidus %
	24 hrs.	48 hrs.		
Vitamine B_1	Bifidus increased	Gram-pos. cocci increased	Soft pH: 5.6	38.0%
TPP	Pure bifidus flora	—	Soft pH: 5.8	70.5%
FAD	Bifidus increased	Pure bifidus flora	Soft	90.1%
Vitamine C	Bifidus increased	Gram-pos. cocci increased. Bifidus decreased	—	38.0%
ATP	Gram-pos. cocci markedly increased	Remained same	Soft pH: 4.6	—

Among the vitamines tested, TPP (Thiamine pyrophosphate) and FAD (Flavin adenine dinucleotide) were markedly active for the growth of L. bifidus. It was of special interest that the microbiological activity of TPP was considerably higher than that of vitamine B_1, in spite of the fact that both compounds belonged to the same vitamine B_1 group. It was beyond my expectation that vitamine C showed only minimal bifidus promoting activity in vivo, in contrast to the in vitro study, in which vitamine C was stolongly active for the growth of L. bifidus in the culture media. Incidentally, the Gram positive cocci showed a marked response to the administration of vitamine C, in vivo.

F. *Injection of Glucosamine Derivative with Lactose into Ileocecum of Infants.*

Table 7. Smears and cultures for L. bifidus upon injection of glucosamine with lactose into ileocecum of infants.

	Smears			Character of stool	Cultures Bifidus %
	24 hrs.	36 hrs.	48 hrs.		
D-glucosamine	Bifidus 30%	Bifidus 20%	Bifidus 30%	Soft	27.0%
β-ethyl-N-Acetyl D-glucosamine	Bifidus 30%	Bifidus 40%	Remained same	Soft	60.0%

Concentration of test materials; 50 mg. of D-glucosamine or β-ethyl-N-acetyl-D-glucosamine was dissolved in each 100 cc. of the basic formula. As indicated in Table 7, β-ethyl-N-acetyl-D-glucosamine showed a moderate increase of L. bifidus but was not potent enough to bring the pure bifidus flora.

In this chapter, has been discussed the growth of L. bifidus when each test material with lactose was administered into the ileocecum of infants. As listed

Table 8. Comparison of activity of organic acid for the growth of L. bifidus between in vivo and in vitro. (−) indicates no growth.

Organic acid	In vitro	In vivo
Pyruvic acid	3(+)	3(+)
α-ketoglutaric acid	4(+)	4(+)
Oxaloacetic acid	(−)	3(+)
Acetic acid	(−)-3(+)	(−)
Citric acid	1(+)	1.5(+)
Lactic acid	(−)	1(+)
Succinic acid	4(+)	4(+)
Fumaric acid	1(+)	4(+)

in the following Tables, a comparison of the microbiological activity of each test material will be made between in vivo and in vitro studies (In vitro study was investigated by Tamaki.[1]). In the Tables, 1(+) or 2(+) represents the grade of the bifidus growth, comparing with the 3(+) which indicates the growth in case of pyruvic acid.

Both α-ketoglutaric acid and succinic acid enhanced the growth of L. bifidus considerably in vivo as well as in vitro, however, the results obtained in using other kinds of organic acid did not always coincide with each other.

In the series of amino acids;

Table 9. Comparison of activity of amino acid for the growth of L. bifidus between in vivo and in vitro.

Amino acid	In vitro	In vivo
Aspartic acid	4(+)	4(+)
Glutamic acid	4(+)	3(+)
Lysine	1(+)	3(+)
Alanine	1(+)	1.5(+)
Glycine	(−)	3(+)
Cystine	4(+)	(−)

As shown in Table 9, the results of both studies (in vivo and in vitro) were parallel in case of aspartic acid and glutamic acid. But lysine was active only in vivo and cystine was active only in vitro.

In case of fatty acid group;

Table 10. Comparison of activity of fatty acid for the growth of L. bifidus between in vivo and in vitro.

	In vitro	In vivo
Propionic acid	4(+)	2(+)
Butyric acid	4(+)	1(+)
Palmitic acid	4(+)	(−)
Linoleic acid	1(+)	(−)

As seen in Table 10, no fatty acid was microbiologically active in vivo, while some was active in vitro. It was remarkable that the Gram positive cocci definitely increased in vivo by the administration of fatty acid.

In case of purine derivatives (Table 11);

Table 11. Comparison of activity of purine derivative for the growth of L. bifidus between in vivo and in vitro.

	In vitro	In vivo
Guanine	4(+)	2(+)
Uracil	(−)	2.5(+)

In case of vitamines (Table 12);

Table 12, Comparison of activity of vitamine for the growth of L. bifidus between in vivo and in vitro.

	In vitro	In vivo
Vitamine B_1	1(+)	(−)
TPP	1(+)	4(+)
FAD	(−)	4(+)
Vitamine C	4(+)	(−)*

* Gram positive cocci increased.

It is of interest that the L. bifidus showed a marked increase in vivo, by the administration of TPP (Thiamine pyrophosphate) or FAD (Flavin adenine dinucleotide). In the case of vitamine C, however, Gram positive cocci increased considerably, while it showed a marked activity in vitro.

In the case of glucosamine derivatives (Table 13);

Table 13. Comparison of activity of glucosamine derivative for the growth of L. bifidus between in vivo and in vitro.

	In vitro	In vivo
D-glucosamine	1(+)	(−)
β-ethyl-N-acetyl D-glucosamine	(−)	1(+)

As shown above, none of the group yielded a remarkable result. Table 14 summarizes the test materials, which were proved to be more biologically active than pyruvic acid, either in vivo or in vitro.

In the in vivo studies, however, there seemed to be relatively a wide range with regard to the rate of the growth of L. bifidus and of the time which was required for establishing their maximum growth. For instance, in case of organic acids, such as pyruvic acid or α-ketoglutaric acid, pure bifidus flora was induced shortly after their administration. But in amino acids or others, it was more de-

layed than in the case of organic acids to have obtained the pure bifidus flora and besides, the Gram negetive bacilli were apt to be still surviving, even when the percentage of colonies of L. bifidus were counted about 70 to 80 percent. It may be, therefore, concluded that the organic acid was most potent for the growth of L. bifidus.

Table 14. Microbiologically active substances for the growth of L. bifidus in vivo or in vitro.

	Organic acid	Amino acid	Fatty acid	Purines	Vitamine
In vivo	α-ketoglutaric A. Succinic acid Oxaloacetic acid	Aspartic A.	—	—	TPP
In vitro	α-ketoglutaric acid Succinic acid	Aspartic A. Glutamic A.	Propionic A. Butyric A. Palmitic A.	Guanine	Vitamin C

Part II. Biochemical Analysis of Ileocecal Content of Infants in Relation to Growth of L. Bifidus.

As stated in Part I, although the various substances which would promote the growth of L. bifidus have been found, there still arose the following questions:

1. Do those substances naturally exist in the ileocecum of infants?
2. What differences were there between the ileocecal contents of the breast fed and of the bottle fed infants?
3. Do those differences give an explanation for the difference of the intestinal flora between breast fed infants and bottle fed infants?

To solve these questions, investigations have been attempted for analyzing ileocecal juice of infants, from these various aspects.

A. *Carbohydrates* (*Especially lactose*)

According to Matsui,[4] average lactose content in the ileocecal juice was 2.1 percent (as the total sugar, 2.3 percent) in case of breast fed infants, compared with 1.0 percent (as the total sugar, 1.2 percent) in the bottle fed infants. By the oral administration of the various concentrations of lactose, ranging from 7 percent to 25 percent, it was found that the lactose absorption in the infant's small intestine appeared to be fairly good, in opposition to the concept that lactose could not be readily absorbed. In fact, the amount of lactose in the ileocecum was always kept in a range of 0.7 to 1.8 percent, as it should be, hence, it is reasonable to assume that there occurs a self-regulating mechanism for controlling sugar concentration in the ileocecal region, so that the concentration may not exceed 2 percent, even if the considerably large amount of lactose would be given orally at a time.

On the other hand, Kuniya[3] found that the sugar concentration should be

maintained at least about 2 percent (1.8 percent as lactose) in order to keep the percentage of L. bifidus in stool over 50 percent. In addition, it was confirmed that lactose was the most biologically active sugar for promoting L. bifidus, of all the carbohydrates tested. Thus, in order to anticipate the predominance of L. bifidus in the alimentary tract of the bottle fed infant, it might be required to maintain the lactose concentration in the ileocecum at least 2 percent, in reference to the fact that its concentration was considerably low in the bottle fed infants. This requirement may be met by introducing any possible means, such as changing the form of lactose in milk or by making the lactose absorption somewhat delayed by introducing any device on the components of milk other than lactose (e. g. protein, fat or salts).

B. *Protein*

Matsuo[5] found that the ileocecal juice of bottle fed infants contained relatively a large amount of total nitrogen (1.5 to 2 times), protein nitrogen (2.5 to 3.5 times) and free amino nitrogen (2.0 to 2.5 times)in contrast to those of breast fed infants.

Petuely[6] emphasized the significance of the protein-carbohydrate ratio of milk as one of the important conditions to induce the predominant bifidus flora, and stated that the ratio should be kept over 1.0 : 2.5, i. e., the requirement of carbohydrate in the ileocecum would be at least twice as much as protein. Table 15 shows the protein-carbohydrate ratio of the milk samples and of the ileocecal contents of infants, fed with the different milk.

Table 15. Protein-carbohydrate ratio of the milk-samples and of the ileocecal contents of infants, fed with the different milk.

		Breast milk	Basic formula*	Milk containing 10% lactose**
Protein-carbohydrate ratio	Milk	1.0 : 5.5	1.0 : 2.0	1.0 : 3.0
	Ileocecal content	1.0 : 1.9	1.0 : 0.8	1.0 : 1.1

* Basic formula: Mixture of 13 percent evaporated milk, 5 percent cane sugar and 2 percent Biocemeal.

** Milk containing 10% lactose: Mixture of 13 percent evaporated milk and 5.5 percent lactose.

From these results, it was found that this ratio could not be easily altered only by a simple procedure such as increasing the amount of sugar in milk. The following study was concerned with the influence of the alteration of this ratio upon the growth of L. bifidus, when the casein solution was administered alone directly into the ileocecum of breast fed infants using the Kobe-Tubing procedure, or when the calcium caseinate-containing breast milk was given orally to them. Namely, the same quantity of milk protein as in cow's milk was scheduled to be

given to the breast fed infants whose protein content in the ileocecal juice was found to be low.

1. *Influence of Injection of Casein into Ileocecum of Breast Fed Infants upon Growth of L. Bifidus*

Examinee

A healthy breast fed infant, 1 month of age, weighing 3.5 kilogram. The infant was fed with 600 cc. of breast milk daily.

Method

Right after breast milk given, each 10 cc. of 3 percent casein solution was injected every 3 hours for the 36 hours period. After the first injection, smears were made every 3 hours and cultures every 9 hours, to find the percentage of L. bifidus in the intestinal flora. PH of the intestinal juice was also examined.

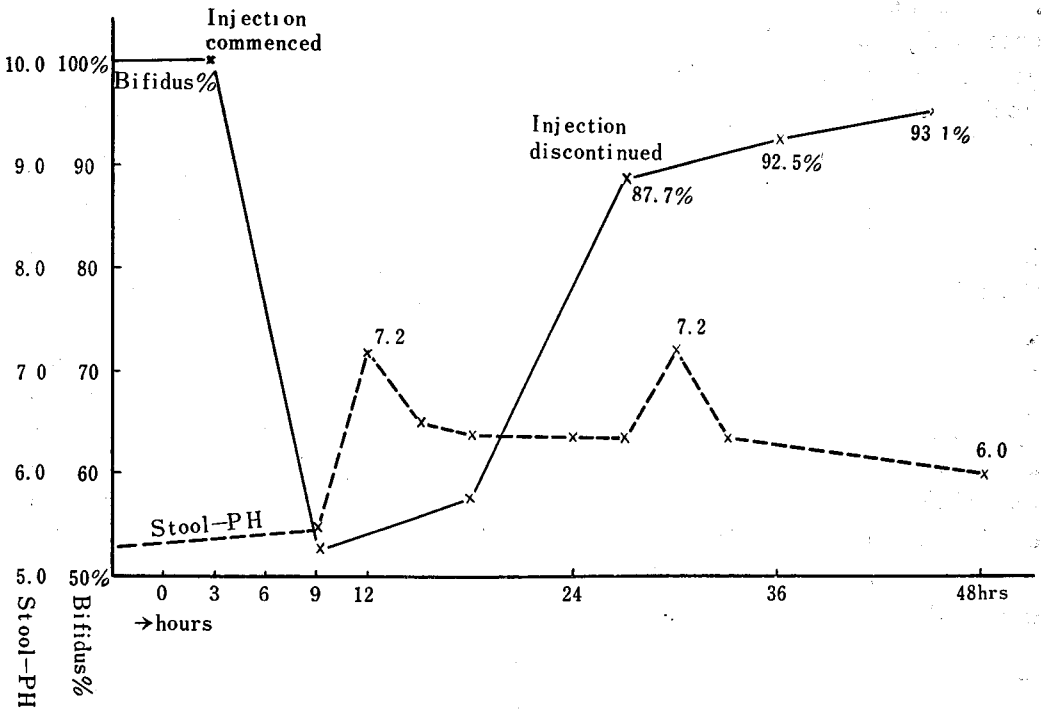

Fig. 1. Percentage of L. bifidus and stool PH of breast fed infant, when each 10 cc. of 3% casein solution was injected into ileocecum every 3 hours.

As seen in Fig. 1, L. bifidus started to decrease about 6 horus after the first injection, and showed a considerable decrease at the 9 hour period (52 percent, PH: 7.2). However, L. bifidus gradually increased and returned to the pure bifidus flora at the 30 hour period, in spite of the continuous administration of the casein solution.

2. *Oral Administration of Mixture of Breast Milk and* 1.9 *percent Calcium Caseinate to Breast Fed Infant.*

Examinee:

A healthy breast fed infant, 1.3 months of age, weighing 4.6 kilogram.

Method:

Mixtures of breast milk and 2.5 percent *Galatosan were given orally per 120 cc. every 3 hours, for 5 days.

*As Golactosan contains 75.8 percent calcium caseinate, 2.5 percent solution of Galactosan contains 1.9 percent calcium caseinate.

Smears and cultures were taken once daily to examine the percentage of L. bifidus in stools. Stool PH was also examined.

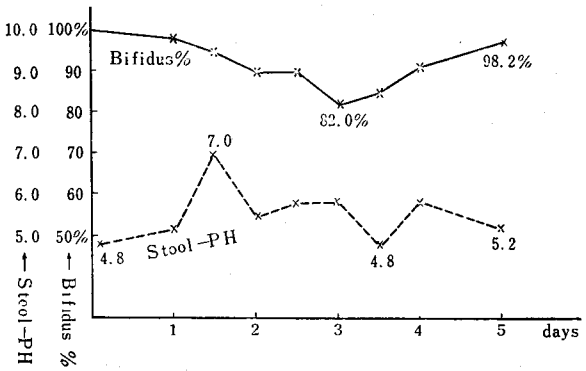

Fig. 2. Percentage of L. bifidus and stool PH of breast fed infant, upon feeding of mixture of breast milk and 1.9 percent calcium caseinate every 3 hours.

Result:

As seen in Fig. 2, a transient decrease of L. bifidus, in relation to a slight increase of stool PH was observed on the second day of this study. But after the third day, the pure bifidus flora was brought just as seen before. From these results, it was concluded that the percentage of L. bifidus and stool PH returned to the previous values, although a transient variation of a slight degree was observed during the period. Thus, it was strongly suggested that the protein-carbohydrate ratio of milk did not always play a great role for the growth of L. bifidus, and that the bifidus flora were scarcely affected even by the stress of the ingestion of the heterogenous milk protein, as long as the breast milk feedings were continued. In addition, it should be emphasized that the microbiologically active substances might be contained in human milk whose potency for enhancing bifidus was considered to be exceptionally great.

C. *Qualitative Determination of Amino Acid in Ileocecal Content of Infants.*

Studies were undertaken on the differences of amino acid in the ileocecal content between breast fed and bottle fed infants by a paperchromatographical technique.

Test Material:

3 cc. of ileocecal juice was deproteinized by the addition of 3 cc. of absolute ethanol and filtered. The filtrates were heated to dryness on the hot water bath (70° to 80°C) and then dissolved with 0.1 cc. of distilled water.

Method:

One dimensional buffer filter paperchromatography devised by Mc Farren[7] (A, B, C, and D system) was used. Butanol-Gracial acetic acid system was also for searching leucine and cystine. The sample solutions were applied to the buffered paper in 0.005 cc. quantities so that the diameter of the spot might not exceed 5 mm. The time for the development of the chromatogram was 20 hours. The developed chromatogram was dipped in the appropriate color reagent (Color reagent No. 1 or No. 2), then heated at 60°C for 15 minutes. For the detection of cystine, Feigl's sodium azide-iodine solution was used as the color reagent and examined under ultraviolet light.

Result:

Incidence of each free amino acid appearing in the ileocecal juice was obtained as shown in Table 16.

Table 16. Incidence of free amino acid in ileocecal juice of breast fed infants and of bottle fed infants.

	Breast fed infant	Bottle fed infant		Breast fed infant	Bottle fed infant
Alanine	*4/8**	13/18	Tyrosine	4/10	6/8
Threonine	4/8	12/18	Phenyl ala.	8/15	14/14
Glycine	4/8	12/18	Proline	9/15	9/14
Serine	5/8	11/18	Arginine	2/15	6/14
Glutamic A.	7/18	18/18	Lysine	13/15	14/14
Aspartic A.	7/8	18/18	Tryptophane	3/6	4/16
Methionine	2/10	3/8	Leucine	3/7	8/10
Valine	6/10	7/8	Cystine	17/18	9/9
Histidine	5/10	3/8			

* Number of cases identified/Number of cases examined **

It was observed that there seemed to be no particular difference of the incidence of each amino acid appearing in the ileocecal juice between breast fed and bottle fed infants. The difference of the bifidus flora between the two types of feeding could not be fully explained from either aspect of protein or amino acid in the ileocecal juice.

D. *Organic Acid*

Comparative studies were made on the difference of organic acid between the ileocecal content of the breast fed infants and of the bottle fed infants. For the qualitative analysis by paperchromatography, Mc Ardle's[8] method and Fukui's[9] method were used for analysing organic acids.

No significant qualitative diference of the organic acid was observed between the ileocecal contents of the breast fed and bottle fed infants. However, it is

striking that both pyruvic acid and α-ketoglutaric acid were present almost constantly in the ileocecal contents. Then the determination of pyruvic acid and total α-ketoacids were made by the Friedemann's[10] method to study whether or not there was any quantitative difference between the contents of the two groups. But as shown in Table 18, there was also no significant differnce by which an etiology of the predominant bifidus flora in breast fed infants could be fully explained.

Table 17. Incidence of organic acid appearing in ileocecal content of breast fed and bottle fed infants.

	Breast fed infants	Bottle fed infants		Breast fed intants	Bottle fed infants
Pyruvic acid	*8/10**	14/17	Butyric acid	0/2	0/2
α-ketoglutaric A.	9/10	15/17	Propionic acid	0/2	0/2
Lactic acid	10/10	10/10	Acetic acid	0/2	1/2
Valeric acid	0/2	0/2	Formic acid	0/2	0/2

* Number of cases identified/Number of cases of examined **

Table 18. Content of α-keto-acids in ileocecal juice.

	Total α-keto-acid	Pyruvic acid
Breast fed infant mg %	8.51	8.21
Bottle fed infant	8.66	7.02

E. *Glucosamine Derivatives*

For the quantitative determinations of free glucosamine and total glucosamine of the ileocecal content of infants, Neuhaus-Letzring's[11] method was used. For determining N-acetyl-D-glucosamine, Aminoff-Morgan's[12] method was used. The samples were deproteinized with the Somogyi's method. Total glucosamine was estimated after hydrolysing samples with 2N-hydrochloric acid for 2 hours.

Result:

Table 19. Free, total and N-acetyl-D-glucosamine (γ/cc) in the ileocecal juice of the breast fed infants.

	Breast fed infants				
	Case 1	Case 2	Case 3	Case 4	Average
Free glucosamine γ/cc	32	40	44	39	36
Total glucosamine γ/cc	1860	1570	2318	1263	1752
N-acety-D-glucosamine γ/cc	—	—	420	550	485

Although there was no remarkable difference in the content of the free glucosamine between the two groups, (table 19 and 20), a significantly larger amounts of total glucosamine and N-acetyl-D-glucosamine existed in the ileocecal content

Table 20. Free, total and N-acetyl-D-glucosamine (γ/cc) in the ileocecal juice of the bottle fed infants.

Bottle fed infants				
	Case 1	Case 2	Case 3	Average
Free glucosamine γ/cc	35	21	21	26
Total glucosamine γ/cc	275	172	262	237
N-acetyl-D-glucosamine γ/cc	140	75	110	108

of the breast fed infants. Takeuchi[13] in our laboratory reported that there was no quantative difference of free glucosamine between the deproteinized filtrates of human milk and of cow's milk, whereas 4 folds of N-acetyl-D-glucosamine and 6 folds of total glucosamine were contained in human milk (Table 21).

Table 21. Free, total and N-acetyl-D-glucosamine contents of human and cow's milk (by Takeuchi).

	Free glucosamine γ/cc	N-acetyl-D-glucosamine γ/cc	Total glucosamine γ/cc
Human milk	40	280	880
Cow's milk	37	70	140

From this aspect, the remarkable difference of the glucosamine content in the ileocecal juice appears to be reflected by the quantitative difference of these substances contained in each original milk. It would seem impossible to give a complete explanation of the predominance of L. bifidus in breast fed infants from the aspect of quantitative differences of total glucosamine and of N-acetyl-D-glucosamine, because of the fact that the pure bifidus flora could not have been induced even by the direct administration of glucosamine compounds with lactose, into the ileocecum of the bottle fed infants.

Neuraminic acid, which has been recognized as one of the most interesting glucosamine compounds, will be discussed also in this chapter. Lactaminsäure-Lactose isolated by Kuhn[14] is composed of lactose, pyruvic acid and N-acetyl-D-glucosamine (Fig. 3). As previously stated, pyruvic acid and lactose were markedly active for the growth of L. bifidus in vitro and also in vivo. Determination of neuraminic acid* in ileocecal content was made by the Böhm's method.[16]

Table 22. Neuraminic acid in the ileocecal contents of breast fed and bottle fed infants.

	Breast fed infant	Bottle fed infant
Neuraminic A. in ileocecal content γ/cc	87	48

*: Neuraminic acid for the standard solution was kindly furnished by Dr. Richard Kuhn.

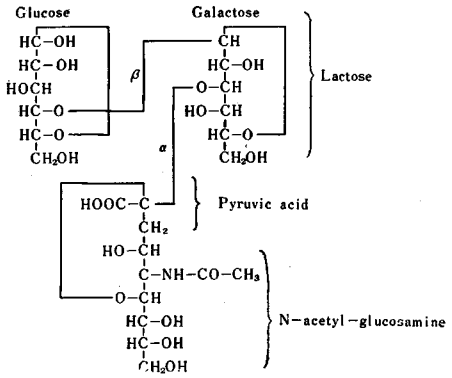

Fig. 3. Lactaminsäure-Lactose (Gottschalk)[15]

Table 22 indicates that the content of neuraminic acid (Bial chromogen-positive substance) in the ileocecal juice of the breast fed infants was much larger than that of the bottle fed infants. Table 23 shows that the neuraminic acid content of the lower part of the small intestine appeared to be increased, compared to that of the upper part.

Table 23. Neuraminic acid content in the various sites of small intestine.

	40 cm.	60 cm.	80 cm.
Breast fed infants γ/cc	30	44	87
Bottle fed infants γ/cc	17	22	46

In short, it was considered that the glucosamine derivatives seemed to be closely related to the growth of L. bifiidus, since there were distinct difference with regard to the glucosamine derivatives between human and cow's milk and also between the ileocecal contents of breast fed and bottle fed infants and besides, Lactaminsäure-Lactose posessed a remarkable activity for L. bifidus.

Conclusion of Part II

Lactobacillus bifidus is a predominant organism seen in the colon of the breast fed infants, being estimated over 90 percent on the stool smears. Many factors might be concerned with the growth of the intestinal flora, but the most imoportant one would be the constituents of the intestinal contents. Qualitative and quantitative evaluation of the main constituents such as carbohydrate, protein, organic acid and glucosamine compounds, were made on the ileocecal contents of both breast fed and bottle fed infants.

1. *Carbohydrate*

Lactose was found to be most active for the growth of L. bifidus. The amount

of lactose was about 1 percent in the ileocecal content of bottle fed infants, in contrast to 2 percent of those breast fed ones. Inasmuch as the growth of L. bifidus would require lactose in the ileocecum at least in concentration of 1.8 percent, it seems to be most unfavorable for the growth of bifidus to have a condition of low lactose with artificial feeding. It is, therefore, highly desirable to increase the lactose concentration in the ileocecal area up to 2 percent in bottle feeding in order to promote the multiplication of this particular organism.

2. *Protein*

The amount of total nitrogen and protein nitrogen in the ileocecal content of the bottle fed infants were both 2 to 3 times greater than those of breast fed babies. The fact that a large amount of protein remained in this area would seem to be unfavorable as far as the thriving of L. bifidus. Milk protein, therefore, should be reevaluated and adjusted to meet the protein requirement of the infant and that the protein content in this area be maintained at a certain low value. In the breast feeding, however, the bifidus flora did not show any alteration in spite of administering the heterogenous cow's milk casein. The result suggests that breast milk is posessed of a high activity for L. bifidus. At present, no adequate explanation for the growth of L. bifidus is available from the standpoint of amino acid, as there is no significant difference between the ileocecal contents of the breast fed and bottle fed infants.

3. *Organic Acid*

Various organic acids were examined for their microbiological activity; however, there was no particular difference in organic acids from qualitative and quantitative view points.

4 *Glucosamine Derivatives*

No quantitative difference was found in free glucosamine between the two infants groups, but the amount of total and N-acetyl-D-glucosamine were both considerably higher in the breast fed group. Pure bifidus flora could not be induced even when a large amount of N-acetyl-D-glucosamine or β-ethyl-N-acetyl-D-glucosamine was administered with lactose, directly into the ileocecum of the bottle fed infants. Thus, it was acknowledged that these kinds of glucosamine deriatives were not directly concerned with the occurrence of the pure bifidus flora. On the other hand, however, it was found that the ileocecal juice of the breast fed infant contained a large amont of neuraminic acid, which was recently advocated as a component of the Bifidus Factor by Kuhn and György.[17] The further studies on the significance of neuraminic acid in relation to the growth of L. bifidus needs to be undertaken.

Part III Discussion and Conclusion on Relationship Between Ileocecal Content of Infants and Growth of L. Bifidus.

As stated in Part I, extensive studies were made on the gowth of L. bifidus

in stool, which could be influenced by the administration of various materials such as organic acids, amino acids, fatty acids, purine derivatives, vitamines or glucosamine derivatives, directly into the ileocecum of healthy infants with the Kobe-Tubing. A clue of this investigation was Kuniya's[2] interesting study that the predominant bifidus flora was successfully obtained by the administration of pyruvic acid with lactose directly into the ileocecum of infants. In the present study, various kinds of organic acid were used instead of pyruvic acid. It was remarkable that α-ketoglutaric acid, oxaloacetic acid and succinic acid were all superior to pyruvic acid in the microbiological activity for L. bifidus. It was of special interest that the combined use of each α-keto acid and lactose showed a considerable microbiological activity in colon of infants, although there were no

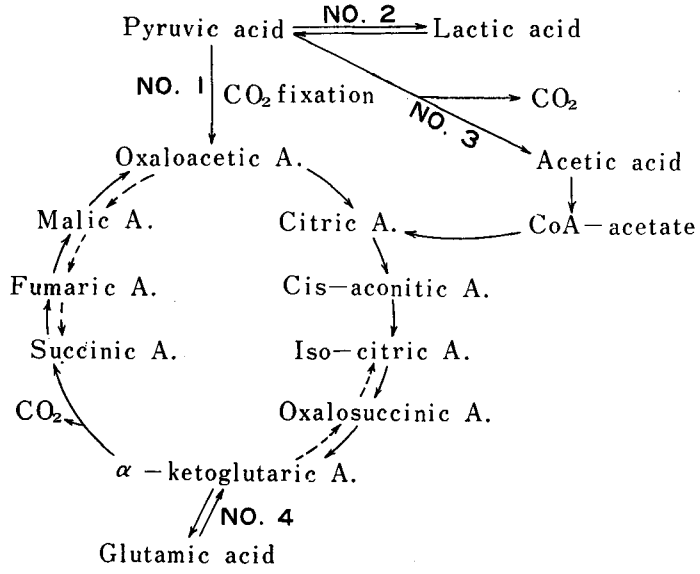

Fig. 4. TCA cycle

particular difference of the incidence of the various organic acids appearing in the ileocecum between the breast fed and bottle fed infants. It has not been settled yet, however, how these organic acids were utilized by the regular strain of L. bifidus. The several conditions may be available referring to the tricarboxylic acid cycle (TCA cycle).

1. *A Pathway from Pyruvic Acid to Oxaloacetic Acid (No. 1 in Fig. 4)*

L. Pine[18] stated that in one strain of L. bifidus, carbon dioxide was fixed in a manner consistent with the operation of the Wood-Werkman reaction with the formation of succinic acid to a minor extent, but that the major pathway in the organism was one which satisfied the equation: Glucose → 3 acetic acid.

2. *A Pathway from Pyruvic Acid to Lactic Acid (No. 2 in Fig. 4)*

R. F. Norris[19] et al reported that the dextrorotatory lactic acid was formed

by the bifid strain and that the inactive lactic acid was formed by the unbranched strain of L. bifidus.

3. *A Pathway from Pyruvic Acid to Acetic Acid (No. 3 in Fig. 4)*

According to Norris et al[19] this route appears to be one of the metabolic pathways of both bifid and unbranched strain of L. bifidus with the formation of volatile acid, presumably of acetic acid. It seems to be of interest referring to the result that the growth of L. bifidus was in a large extent accelerated by the administration of thiamine pyrophosphate (TPP), which was considered to be a coenzyme of this reaction.

4. *A Pathway from α-ketoglutaric Acid to Glutamic Acid (No. 4 in Fig. 4)*

The bifidus promoting activity of α-ketoglutaric acid was found to be pronouced by the present study. Miwa[20] found that an amination reaction was available in L. bifidus like the following;

$$\alpha\text{-ketoglutaric acid} + NH_3 \rightleftarrows \text{Glutamic acid}$$

5. *Transamination Reactions*

Kuniya and Miwa[20] demonstrated the presence of transaminases; glutamic-pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) in L. bifidus.

$$\text{Pyruvic acid} + \text{Glutamic acid} \rightleftarrows \alpha\text{-ketoglutaric A.} + \text{Alanine}$$

$$\text{Oxaloacetic acid} + \text{Glutamic A.} \rightleftarrows \alpha\text{-ketoglutaric A.} + \text{Aspartic A.}$$

So, it might be possible to imagine that the protein synthesis of L. bifidus could be performed by these reactions. It seems to be difficult to decide whether or not the metabolism of L. bifidus was performed along the TCA cycle by utilizing organic acids, but it is likely that all the courses composing the TCA cycle were not available as a whole, since none of the strain of L. bifidus produced carbon dioxide and that not every substance, which belonged to TCA cycle showed the bifidus promoting activity either in vitro or in vivo.

With regard to amino acid, aspartic acid, glutamic acid and lysine were potent in order. Cystine was inactive in vivo in contrast to in vitro. It was indicated by J. B. Hassinen[21] that cystine or cysteine was the only amino acid whose removal from the medium resulted in decreased growth.

In reference to fatty acid, R. M. Tomarelli et al[22] stated that the growth factor of milk was found to be associated with the unsaturated fatty acids, such as oleic and linoleic acid and related compounds also exhibited growth stimulation. The present study, however, indicated that the fatty acids tested, were found all inactive in vivo.

With regard to the series of purine and nucleic acid, R. M. Tomarelli[22] reported that the compounds containing a desoxyriboside component were found to be active for the variants of L. bifidus. According to Kitay et al,[23] thymidine, hypoxanthine desoxyriboside, adenine desoxyriboside or cytosine desoxyriboside were equally active in promoting growth and their effects in growth stimulation

did not seem to be specific but dependent on the desoxyriboside structure. In the present study, uracil was found active in vivo.

As to the glucosamine derivatives, it was found by P. György[24] that the growth factors in human milk for L. bifidus var. Penn consist of a group of N-acetylglucosamine-containing oligo- and polysaccharides. E. Walch[25] reported that bifidus flora increased up to 65 percent upon the oral administration of β-ethyl-N-acetyl-D-glucosaminide in concentration of 50 mg. per 100 cc. of cow's milk. Meanwhile, Goya[26] observed that a certain significant alteration of the percentage of the bifidus flora was not obtained in the similar studies. The present study indicates that there was no definite activity of this substance when it was injected into the ileocecum.

It was demonstrated by Zilliken F.[27] that a nearly selective synthesis of microbiologically active 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine had been achieved by incubation of intact cells of L. bifidus var. Penn with lactose and N-acetyl-D-glucosamine. It is implied by this result that lactose and glucosamine compounds may be closely related to the Bifidus Factor as their precursor. In recent years, Kuhn[28] obtained fucosidolactose, various N-containing oligosaccharides and acidic oligo- and polysaccharide (N-acetyl-neuraminic acid) from human milk, which were found microbiologically active. These substances are all closely related to lactose or N-acetyl-D-glucosamine in view of their structure. It is of special interest that in the present study, N-acetyl-D-glucosamine and total glucosamine were found to be rich in human milk and also in the ileocecal content of the breast fed infants, compared to those of the bottle fed.

It has been established that lactose was best utilized by L. bifidus. This fact was confirmed by the Kuniya's[2] study that the lactose was shown definitely superior in the bifidus promoting activity in vitro as well as in vivo. It may be therefore, considered that lactose would play a special role in enhancing the growth of L. bifidus, not only being utilized as an energy source but also being available because of its structure. It was, of course, impossible to obtain the pure bifidus flora with the single administration of lactose. But the bifidus promoting activity of lactose was proved to be potent, when the various substances were added to to lactose.

The mechanism of the growth of L. bifidus will be discussed, by summarizing the results obtained in the present study in which the comparison was made between the ileocecal content of the breast fed and bottle fed infants.

As illustrated in Fig. 5, when it is assumed that lactose, α-keto-acids, and glucosamine are indispensable for the growth of L. bifidus, it would seem that human milk thoroughly contained those three substances (Fig. A) and the best result has been obtained when those three substances were well fulfilled, even in aerobic culture media (Fig. B). As it was apparent that cow's milk contained insufficient amount of lactose and glucosamine (Fig. C), an attempt was made to supply those substances to meet a deficit, aiming at obtaining the pure bifidus

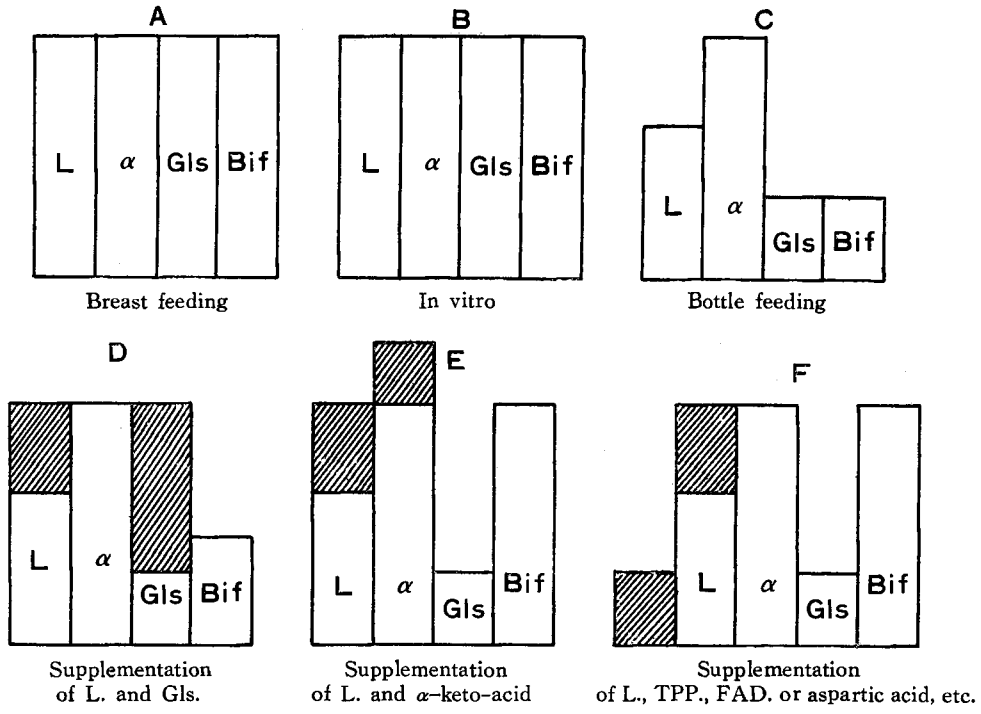

Fig. 5. Correlatien of growth stimulating effect for L. bifidus among lactose, α-keto-acids and glucosamine.
L: Lactose Gls: Glucosamine α: α-keto-acids Bif: L. bifidus

culture. But it ended in a failure (Fig. D). In contrast, the combined use of lactose and α-keto-acids (Fig. E), TPP, FAD or amino acids such as aspartic acid and lysine (Fig. F) resulted in promoting growth of L. bifidus.

Until recently, no practical metod has been known successful for the purpose of inducing the pure bifidus flora except for feeding infants with breast milk. In other words, it has been considered that the microbiologically active substance existed only in human milk. In the present study, it has become possible to induce the predominant flora of L. bifidus just as seen in breast fed infants, even if neither breast milk nor special substances such as Bifidus Factors (isolated by Kuhn and György) would be used for infants at all. The mechanism of the growth of L. bifidus, of course, still remains to be clarified; however, it is of special interest to have succeeded in obtaining the pure bifidus flora in vivo, by the direct administration of the various kinds of material other than the breast feeding.

SUMMARY

Biochemical and clinical investigations were made on the mechanism of the growth of Lactobacillus bifidus in infants.

1. Various materials which were known microbiologically active for L. bifidus in

vitro, were chosen and were injected with lactose into the ileocecum of bottle fed infants to observe whether or not the growth of L. bifidus could be induced. α-ketoglutaric acid, succinic acid, oxaloacetic acid, pyruvic acid, aspartic acid and thiamine pyrophosphate were proved to be markedly active with the result that the same pure bifidus flora was obtainable as seen in breast fed infants. No side effect was observed.

2. Biochemical analysis was made on the ileocecal content of breast fed and bottle fed infants. The correlation between the constituents and the growth of L. bifidus was also studied. For accelerating the growth of L. bifidus, the following conditions with regard to the ileocecal content appeared to be important.

a. Lactose concentration should be ranged at least in about 2 percent.
b. Excessive amount of protein should not be present.
c. Certain glucosamine compounds seemed to be indispensable.

Amino acid and organic acid were not particularly concerned with the growth of L. bifidus from both qualitative and quantitative view points.

3. The mechanism of the growth of L. bifidus was discussed in relation to the microbiological activity of the various substances. Lactose, α-keto-acids and glucosamine derivatives were considered to be the major factors required for the bifidus growth. At present, an attempt at summarizing the mechanism of the growth of L. bifidus does not seem to have been accomplished yet; however, it is of special interest to have succeeded in obtaining the pure bifidus flora in vivo, by the direct administration of the various kinds of material aside from breast milk, using the Kobe-Tubing procedure.

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