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KUNIYA, Terumichi

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Terumichi KUNIYA

(Received for publication 3, June 1959)

It has been well established that breast-fed infants could be more favored in their growth and development and seemed to be more resistant against infection than bottle-fed infants, and for this reason the breast feeding has been accepted as the best and ideal. Then, what is the advantage of breast feeding which is superior to the bottle one? For what constituents should cow's milk be modified to adapt human nursing? Studies on these questions are vital problems in the pediatric field and are considered to be increasingly important because of the recent prevalence of artificial feeding. These, of course, have been studied by many investigators and numerous reports have been published, but the results were far from perfection.

It has been demonstrated that the differences between breast- and bottle-fed infants were revealed in condition of nutrition, growth and development, and tendencies in susceptibility of disease. But, one of the most striking differences is found in the character of the stool. Namely, the stool of the breast-fed infant is yellow to golden in colour, acid in reaction (pH ca. 5.0), acid in odor, and *Lactobacillus bifidus* is predominant in the intestinal flora (over 90 percent). On the contrary, the stool of the bottle-fed infant is brown in colour, tends rather to alkaline in reaction (pH ca. 7.0), stinking in odor, the intestinal flora is complex, and *L. bifidus* occupied only 20 percent at highest of total bacterial counts in the stool (so-colled mixed flora).

Adam (1) and Bessau (2) have pointed out that there might be some relation between this overwhelmingly prevalent *bfidus*-flora and the superiority of breast feeding. Accordingly, it is considered that the rate of *L. bifidus* in stool might be able to be an arbiter of the degree of humanization of artificial feeding, and many studies on culture or calculation methods of *L. bifidus* have been reported and a number of attempts also have been done to make the organism prevail in stool by many varieties of modified artificial feedings.

An attention to the difference of composition between human and cow's milk, has been paid for decades, especially of their carbohydrate contents, and the addition of lactose to an artificial feeding has become a matter of controversy. Thus, various types of lactose, for instance, α-lactose (3), caramerized sugar (3), or β-

国屋輝道
From the Department of Pediatrics, Kobe Medical College (Director: Prof. Yoshiho HIRATA)

lactose (4, 5, 6, 7) were tried and all these methods have been more or less successful for the development of *bifidus*-flora.

There is no clear explanation, however, why *L. bifidus* prevail in the stool by adding lactose to artificial feeding. In general, it is accepted that lactose is poorly absorbed from the infant intestine (8, 9, 10), and a portion of lactose escaped from decomposition or absorption reaches the lower part of the intestine, making an optimum condition for the organism to grow. The explanation, however, is merely speculative and is not based on the decisive evidence.

Using Kobe-Tubing procedure (11), which is elaborated in our laboratory, it was possible to collect specimens of the intestinal contents or to transfuse any material directly into the infant intestine at any level. By this method, the author had a good fortune to estimate the amount of sugar in the intestinal contents and its relation to the intestinal flora, and to investigate the influence on the intestinal flora of various sugars introduced directly into the ileocaecal region. With artificialy fed infants the author had succeeded to develop the pure *bifidus*-flora similar to that of breast-fed infant by simultaneous administration of lactose and pyruvic acid directly into the intestinal canal. The present paper summarizes the results of these studies.

EXPERIMENTAL

I. *Relation between Sugar Concentration of the Ileocaecal Contents and the Percentage of L. bifidus in Stool.*

Using transintestinal intubation (Kobe-Tubing) the intestinal contents were aspirated directly from the ilc.* region of healthy infants who were fed on various kinds of diet and the relation between the sugar concentration of the contents and the percentage of *L. bifidus* in the bacterial count of the stool was observed.

1. Method.
   a. Examinee: Healthy infants of 3 to 6 months of age.
   b. Diets:
      (1) Evaporated cow's milk; The evaporated milk diluted at the ratio 2 : 3 was employed as whole cow's milk (whole EM).
      (2) Basal formula (BM); Whole EM plus 4.5 percent dextri-maltose and 1 percent cereal.
      (3) Lactose formulae (L); Milks containing lactose of various concentrations were employed. Since the whole EM contained lactose of 4.5 percent, twice diluted whole EM was called 2.25 L, and milks which were called 7L 10L and 15L mean that were added with lactose of 2.5, 5.5 and 10.5 percent respectively to the whole EM. And also milks which were called 20L and 25L mean those added with lactose of 17 and 22 percent respectively to a 2 in 3 diluted whole EM. Namely, each figure before L indicated the

* The following abbreviation will be used hereafter: ileocaecal = ilc.

total concentration of lactose in the milk employed.

(4) Dextri-Maltose formulae (DM); Similarly in case of lactose formulae, dextri-maltose was used as added sugar. Milks called 7DM and 15DM mean those which were added with dextri-maltose at the rate of 2.5 and 10.5 percent respectively to the whole EM. And milks called 20DM and 25DM mean those added with dextri-maltose of 17 and 22 percent respectively to a 2 in 3 diluted whole EM. Therefore, milks of this group contained both lactose and dextri-maltose as sugar, for example, 25DM contained 3 percent lactose and 22 percent dextri-maltose.

(5) Human milk formulae (HM); Intact or variously modified human milk were employed. So, 1/3HM means a 1 in 3 diluted human milk, (1/3HM+5L) 1/3HM plus 5 percent lactose, and (HM+5L) undiluted human milk plus 5 percent lactose.

c. Plan of Experiment.

As the 4th and 6th day from the beginning of experimental feeding, the intestinal contents were aspirated from the ilc. region, after confirming that the intestinal flora was stabilized, and the concentration of sugar in the contents was measured.

d. Methods of Aspiration of Intestinal Contents from the Ileocaecal Region of the Infant Intestine.

The hole of the tube was placed at the ilc. region and the minimal aliquot of the intestinal contents (ca. 1.0 ml.) was aspirated with a syringe.

e. Determination of Sugar Goncentration and Fractionation of Loctose.

Concentration of sugar was determined by anthrone method (12), and lactose fraction was determined after separation by paperchromatography (13).

f. Culture Method of the Intestinal Flora.

The medium shown in Table 1 was employed for culture of the intestinal flora. A mass of stool directly taken from the anus of infants with a glazed sterile tube was diluted with physiological saline so appropriately as about one hundred colonies might be obtained after culture. Of all colonies, which were grown after 48 hours' incubation at 37° C aerobically by deep tube shake method, Gram-stained (Hucker's modification) slide films were prepared and *L. bifidus* was differentiated microscopically from other bacteriae.

Table 1. Medium for Aerobic Culture of the Intestinal Flora.

Casein Hydrolysate	10.0 ml.
Yeast Extract	0.5 gm.
Peptone	5.0 gm.
Lactose	30.0 gm.
Tomato Juice	300.0 ml.
Cystein	5.0 gm.
Pyruvic Acid	10.0 ml.
Tween 80	0.5 ml.
Pancreatin	0.5 gm.
Agar Agar	20.0 gm.

add. water to 1,000 ml.
Adjust the pH to 6.8 with N/10 NaOH and autoclave at 120 °C for 10 minutes.

At the same time, character, pH (Toyo-Roshi pH-test paper) and smears of the stool were investigated twice a day, daily, observing carefully condition of the infants, amounts of diet consumed and gain in weight.

2. Results (Table 2).

a. Group of Lactose Formulae: Both average and maximum sugar concentrations of the ilc. contents increased according to the amount of lactose added in the formulae, till the case of 15L. But, when 20L or 25L was fed, the contents showed no increase despite addition of large amounts of lactose than before. While, the rate of *L. bifidus* in stool gradually increased in accordance with elevated sugar concentration of the ilc. contents and the rate reached ca. 50 percent on 15L. But, even with increased amount of lactose over 15L, the rate of *L. bifidus* failed to increase over 50 percent; on the contrary, there was a tendency rather to decrease.

b. Group of Dextri-Maltose Formulae: Like in the group of lactose formulae, the values of both average and maximum sugar concentrations in the ilc. contents increased according to the amount of added sugar. But, when the total sugar content of the diet was equal to that of lactose group, the average sugar concentration in the ilc. contents was lower and also the rate of *L. bifidus* in stool was remarkably lower than those of the group of lactose formulae, for instance, it merely reached ca. 21 percent on 25DM.

Table 2. Sugar Concentration of the Ileocaecal Contents and Percentage of *L. bifidus* in Stool.

a. Lactose Formulae.

		2.25 L	7 L	10 L	15 L	20 L	25 L
Sugar	No. of cases	3	8	2	8	5	5
	Average total sugar %	0.7 (0-1.9)	1.1 (0.1-2.1)	1.6 (0.8-2.5)	2.3 (0.3-4.4)	2.2 (0.4-4.3)	2.4 (0.4-4.8)
	Lactose %	-	0.7	1.2	1.8	—	—
L. bifidus	No. of cases	—	3	2	4	3	5
	Average %	—	19.6 (13-25)	31.4 (28-34)	53.0 (45-63)	37.3 (25-47)	40.4 (32-54)

b. Dextri-Maltose Formulae.

		7 DM	15 DM	20 DM	25 DM	BM
Sugar	No. of cases	7	7	5	5	3
	Average total sugar %	1.3 (0.8-2.1)	1.4 (0.3-2.3)	1.5 (0.3-4.0)	1.7 (0.6-4.8)	1.5 (0.7-2.4)
	Lactose %	—	—	—	- -	1.2
L. bifidus	No. of cases	5	5	3	4	3
	Average %	7.8 (2-14)	13.6 (6-23)	19.8 (15-24)	21.7 (11-31)	7.8 (0-13)

c. Human Milk Formulae.

		1/3 HM	1/3 HM+5L	HM	HM+5L
Sugar	No. of cases	1	1	9	1
	Average total sugar %	1.4 (0.7-2.0)	1.6 (1.4-2.0)	2.3 (0.5-4.6)	2.2 (1.5-3.1)
	Lactose %	—	—	2.1	—
L. bifidus	No. of cases	2	2	2	—
	Average %	54.1 (46-55)	69.5 (61-76)	83.8 (78-88)	—

c. Group of Human Milk Formulae: With human milk, the total sugar concentration in the ilc. contents was 2.3 percent on an average and 4.6 percent on maximum, which showed nearly the same level as 15L of the lactose group. It is reasonable that the average and maximum sugar concentrations in the ilc. contents decreased to 1.4 percent and 2.0 percent, respectively, when diluted human milk such as 1/3HM was administered. It is interesting, however, that when diluted human milk was strengthened in the sugar content equal to the original milk by adding lactose (1/3HM + 5L), the values of average and maximum sugar concentrations decreased to 1.6 percent and 2.0 percent respectively, which were far lower than that of undiluted human milk. Moreover, when the strengthened human milk with 5 percent lactose, namely (HM + 5L), was administered, both average and maximum sugar concentrations of the ilc. contents tended to decrease to 2.2 percent and 3.1 percent respectively, contrary to our expectation.

In regard to the intestinal flora, when fed on diluted human milk (1/3HM), the percentage of *L. bifidus* in the stool was distinctly low (54.1 percent), but, even when lactose was added to 1/3HM and the sugar content of the milk was made to be equal to that of undiluted human milk (1/3HM + 5L), the *bifidus* percent in the stool was lower (69.5 percent) than that of undiluted human milk (83.8 percent).

d. Relation between Lactose Concentration of the Ileocaecal Contents and Percentage of *L. bifidus* in the Stool: The *bifidus* percent in stool in relation to total sugar concentration in the ilc. contents was described as above, and that in relation to concentration of lactose fraction is also shown in Table 2. As shown in the table, it was found that the concentration of lactose fraction in the ilc. contents should be over 1.8 percent in order to get *bifidus* percent in the stool over 50 percent, and when the lactose concentration was under 1.8 percent the *bifidus* percent in stool would not reach 50 percent level, as seen in case of 7L or BM.

3. Conclusion.

Various experimental formulae have been tried in the feeding of healthy infants of 3 to 6 months of age and the concentrations of total sugar and lactose fraction in the ilc. contents were determined, and at the same time the rate of *L. bifidus* in stool was estimated by culture.

In the group of lactose formulae, both average and maximum sugar concentrations in the ilc. contents increased gradually from 2L to 15L, and the percent of *bifidus* in the stool also increased according to the amount of added lactose. But, even when an amount of lactose increased over 10.5 percent (15), there was no increase in the total sugar concentration and the percent of the organism in the stool tended rather to decrease less than that of 15L. Consequently, it was assumed that there seemed to be a self-regulating mechanism in the alimentary tract of infant as regards to the concentration of sugar in the ilc. contents, and the percentage of *L. bifidus* in the stool might not be able to increase over 50 percent by adding lactose alone to the diet.

In the group of dextri-maltose formulae, both average and maximum sugar concentrations of the ilc. contents showed extermely low level, as compared to those of the group of lactose formulrae. Namely, the sugar concentration in the ilc. contents varied according to the kind of the sugar added, even though the amounts of sugar were the same.

In the group of human milk formulae, it would be reasonable that the amount of total sugar of the ilc. contents decreased distinctly when fed on 1 in 3 diluted milk, however, it was lower than that of undiluted human milk, even when fed on (1/3HM+5L), inspite of the presence of lactose in the diet of the same amount as in the original milk. When fed on (HM+5L), namely, when lactose content alone was increased over that of human milk, the total sugar contents showed rather lower figure than that of the human milk alone.

In regard to *bifidus* percent in the stool, of course, *L. bifidus* was predominant in number and regular in shape in the group of undiluted human milk. When fed on 15L, in which case the total sugar concentration in the ilc. contents was nearly equal to that of the group of undiluted human milk, *bifidus* percent presented 53 percent, which was far lower than that of human milk, and decreased gradually according to the decrease of lactose content of the milks administered. On the other hand, in the group of dexri-maltose fomulae, maximum value of *bifldus* percent in stool was less than 20 percent, that is, in the promotion of the development of *bifidus*-flora the action of lactose was superior to that of dextri-maltose and the percentage of *L. bifidus* was related to the increased amounts of sugar in the ilc. contents.

Hereupon, two points must be stressed. The first is that a certain condition was required in respect to the existence of sugar in the ilc. contents qualitatively and quantitatively, in order to maintain the *bifidus* percent in stool over 50 percent level, namely, (1) the amounts of total sugar in the ilc. contents were required to be more than ca. 2.0 percent; (2) lactose molecule itself was necessary as the form of sugar in the ilc. contents.

Concerning with the relation between the percent of *L. bifidus* in stool and the amounts of lactose in the ilc. contents, when fed on 7L (0.7 percent as lactose fraction) or BM (1.2 percent), the percentages were low, but when fed on 10L (1.2 percent), 15L (1.8 percent), or HM (2.1 percent), the percentage gradually increased to 31.4, 53 or 83.8 percent, respectively, according to the increase of the lactose fraction in the ilc. contents. This finding suggested that the multiplication of *L. bifidus* is closely related not only to the sugar contents, but also to the formula of sugar in the ilc. contents. That is, in order to maintain the percent of *bifidus* in stool over 50 percent, the sugar concentration in the ilc. contents was required to be at least 2.0 percent as total and 1.8 percent as the lactose fraction. Thus, these findings suggest vital necessity of lactose in the ilc. contents in order to develop the *bifidus*-flora.

The second point is the fact, that, when fed on a 1 in 3 diluted human milk, the percentage of *bifidus* in stool remained on about 50 percent level, and when 5 percent lactose was added to this diluted human milk, the increase of the percentage of *L. bifidus* was minimum. It was suggested, therefore, that human milk did contain, besides lactose, the essential factor for the development of *bifidus*-flora.

II. *Changes in the Intestinal Flora and the Character of Stool by Direct Administration of Various Sugars into the Ileocaecal Region.*

For the promotion of *L. bifidus* in the intestinal flora, the existence of relatively large amounts of lactose in the ilc. region seemed to be imperative, which was proved to be difficult to attain by solely oral route; hence, a procedure has been attempted, by which various types of sugar could be introduced by direct local administration into the ilc. region and which would furnish us information on the acceleration of the *bifidus*-flora as well as an influence on the stool.

1. Method.

a. Examinee and Culture of Stool: Same as previously described.

b. Diet: Basal formula (BM).

c. Procedure of Direct Administration of Sugar into the Ileocaecal Region:

The small opening of the tube was placed at an approximate location of the ilc. region and the various sugar solutions were transfused with a syinge through the oral end of the tube.

d. Kinds and Amounts of transfused Sugar Solutions: An aliquot of 10 ml. of 20 percent aqueous solutions of various sugars, such as lactose, maltose, sucrose, glucose, galactose, and fructose, were transfused three times a day (morning, noon, evening) or 8 times a day for every 4 hours. The treatment was continued for 3 days and the stool was cultured on the 4th day.

e. Finding of Stool: Macroscopic observation of the stool, frequency as well as the character, was noted. Diarrhoea is, then, defined as any types of loose bowels or frequent defecations (6 to 7 times a day).

2. Results.

a. Results of the transfusion of various sugars three times a day (Table 3).

Provided that the effectiveness of various sugars in accelerating the growth of *L. bifidus* was defined to be positive when the rate of the microorganism in stool reached 50 percent level, it has been shown that 5 out of 12 cases of lactose, 2 out of 7 of galactose, and 1 out of 4 of fructose were positive. Thus, lactose

Table 3. Incidence of Increase of *L. bifidus* upon Transfusion of Various Kinds of Sugar into the Ileocaecal Region of Infants (thrice a day)

Kinds of Sugar	*Bifidus* % before Transfusion (in Average)	Incidence of Increasing *L. bifidus* to about 50%	Incidence of Diarrhoea
Dextrin	29.0	None of 4 cases	None of 4 cases
Lactose	16.6	5 out of 12 cases	4 out of 12 cases
Maltose	21.9	None of 6 cases	None of 6 cases
Sucrose	3.8	None of 6 cases	1 out of 6 cases
Glucose	14.3	None of 8 cases	3 out of 8 cases
Galactose	15.2	2 out of 7 cases	None of 7 cases
Fructose	17.5	1 out of 4 cases	2 out of 4 cases

was most effective in promoting the *bifidus*-flora, followed by galactose and fructose. With smear examination of stools of positive cases, Gram-positive cocci and bacilli increased in number; these findings were also confirmed by culture. On the other hand, when large amounts of sugar were transfused, diarrhoea was likely to occur. Although loose bowels were found in 4 out of 12 cases of lactose, 1 out of 6 of sucrose, 3 out of 8 of glucose and 2 out of 4 of fructose, they were of mild; none was to have to discontinue the administration since infant appetite or gain in weight was unaffected.

b. Results of lactose transfusions for every 4 hours (Table 4).

As lactose was the most effective sugar for the gowth of *L. bifidus* as shown above, 7 transfusions of lactose were continued for every 4 hours in 6 cases and

Table 4. Incidence of Increase of *L. bifidus* upon Transfusion of Lactose into the Ileocaecal Region of Infants. (Every 4 hours, after 7 transfusions)

No.	*Bifidus* Percentage		No.	*Bifidus* Percentage	
	Before Transfusion	After Transfusion		Before Transfusion	After Transfusion
1	4.3	43.7	4	6.6	39.4
2	11.3	32.6	5	2.3	27.9
3	5.8	44.8	6	3.6	13.7

the culture of the stool was performed 2 hours later after the last transfusion, under consideration that *bifidus*-flora would be brought on by transfusing large amounts of lactose directly into the ilc. region. The results are shown in Table 4, in which the maximum value of *bifidus* percent in stool was 44.8 percent and minimum 13.7 percent. Thus, it was found that even if large amounts of lactose were transfused directly into that region, lactose alone could not make *L. bifidus* prevail in stool and the ability of the sugar for promoting *bifidus*-flora would be limited, to the extent of about 50 percent in stool.

3. Conclusion.

It was found that only lactose and galactose showed good effect of promoting *bifidus*-flora among the kinds of sugar tested, and that, it was suggested that *L. bifidus* would not be expected to prevail in stool over 50 percent even how high the concentration of lactose in the ilc. contents might be achieved.

It has been generally accepted that lactose was the most effective carbohydrate for increasing the percent of *bifidus* in stool, because of the poor absorption from the small intestine of infant. This conception, however, should be re-evaluated, because the poor absorbability of lactose was denied as in the former chapter and also because of the fact that response of *L. bifidus* to the increasing sugar contents might be limited only to some extent as seen in this chapter.

III. *Multiplication of L. bifidus in vivo upon Transfusion of Pyruvic Acid With or Without Combined Use of Lactose into the Ileocaecal Region of Infant.*

1. Administration of Pyruvic Acid alone.

It has been demonstrated that the pure *bifidus*-flora commonly found in the stool of breast-fed infant could not be obtained even with large amounts of various sugars administered directly into the ilc. region. Dr. Tamaki of our department had proved that pyruvic acid had the power to allow *L. bifidus* grow aerobically *in vitro*, and the acid was tested for possible *bifidus*-flora promoting action *in vivo*.

a. Method.

(1) Examinee, Feeding, Administration Procedure and Stool Culture: Similar to these described previously.

(2) Transfused Substances and the Quality: An aliquot of 10 ml. of 0.5 percent aqueous solution of pyruvic acid was transfused thrice a day, for 3 consecutive days.

b. Results.

During and after the transfusion, Gram-positive bacilli were observed on smears of stool twice a day, but, no increase in the percentage of Gram-positive bacilli could be confirmed, and no abnormal signs were observed.

2. Joint Administration of Pyruvic Acid With Lactose.

As the expected result could not be obtained by pyruvic acid alone, it had to be combined with lactose, because the latter in the ilc. region of the infant fed on the basal formula was considered to be too little to obtain the pure *bifidus*-flora in stool.

a. Method.

(1) Administration: An aliquot of 10 ml. of mixture containing the equal parts of aqueous solution of 0.5 percent pyruvic acid and 20 percent lactose was

transfused every 4 hours continuously to four infants.

(2) Other methods employed: Similar to these previously described.

b. Results (Table 5).

Results showed the development of the pure *bifidus*-flora and the character of the stool also resembled to that of breast-fed infant, all cases tracing the similar courses. Eight hours after the administration, the stool of these infants have changed to acid in reaction (pH 5.8), light yellow in colour, acid in odor, soft or watery in consistence, and the extreme increase of Grame-positive bacilli was observed. With

Table 5. Simultaneous Administration of Pyruvic Acid Together with Lactose into the Ileocaecal Region.

	Percentage of *L. bifidus* in stool	Character of Stool		
		Colour	Consistence	pH
Before Transfusion	6.1%	yellowish white or light yellow	soft or hard	7.4
8 hours after Transfusion	Gram-positive bacilli gradually increased and Gram-negative bacilli decreased	light yellow	soft or loose	5.8
16 hours after Transfusion		light yellow or yellow	soft or watery homogeneous	5.2
24 hours after Transfusion		yellow	soft or watery homogeneous	4.6
32 hours after Transfusion	83.0%	yellow or yellowish brown	soft or watery homogeneous	4.6

the lapse of time, these Grame-positive bacilli multiplied enormously, and by 32nd hour, the pH of the stool fell to 4.6 and the smears stained by Gram's method became microscopically identical to that of breast-fed infants, which Gram-positive bacilli occupied almost all the fields of microscope and in several places groups of few Gram-negative bacteriae were seen (Fig. 1 and 2).

By culture at this time, the average value of *bifidus* percent in the stools of all 4 cases showed up to 83 percent, which was almost equal to that of breast-fed infants.

3. Conclusion.

When pyruvic acid alone, which was able to make *L. bifidus* grow aerobically *in vitro*, was administered into the ilc. region, there occurred no increases of the organism. It has been assumed that the average value of lactose contents in the ilc. region was too low to expect the pure *bifidus*-flora when fed on basal formula, but, when the mixture of pyruvic acid and lactose was administered to offer an ample supply of this deficient sugar, it was possible to produce the *bifidus*-flora as shown in Fig. 2, which presented complete appearance of the intestinal flora of breast-fed infants.

IV. *Multiplication of L. bifidus upon Administration of Pyruvic Acid Together With Various Sugars except Lactose.*

When pyruvic acid was administered into the ilc.region together with lactose,

Table 6. Percentage of *L. bifidus* upon Administration of Various Sugars in Combination with Pyruvic Acid into the Ileocaecal Region.

10 ml. of solution containing 0.5% pyruvic acid and 20% sugar was transfused for every 4 hours. Cultured after the 32nd hour.

Kinds of Sugar	No. of Cases	*Bifidus* Percentage
Glucose	1	44
Galactose	1	29
Glu+Gal.*	1	27
Fructose	1	68
Sucrose	1	65
Maltose	1	Not increased
Dextrin	1	Not increased
Lactose	3	83

* Glu+Gal.=equimolar mixture of glucose and galactose

pure *bifidus*-flora has been obtained in a short time, as mentioned above. Then, what will happen if pyruvic acid was given with other sugars ?

1. Method.

Sugars administered: Dextrin, maltose, fructose, glucose, galactose, and equimolar mixture of glucose and galactose were used as sugar. Method of preparation of the sugar solutions and others are similar to those already described.

2. Results (Table 6).

Of various sugars tried, lactose was proved to be most effective for the growth of *L. bifidus*, followed by fructose, sucrose and glucose in order, galactose or equimolar mixture of glucose and galactose was less effective, and dextrin and maltose were entirely ineffective. But, even in effective cases, the stools were not so similar to that of breast-fed infants as in case of lactose, and the differences were revealed in the time required to attain maximum *bifidus* percent in stool, or pH, character, consistence and odor of the stool.

It is interesting that *L. bifidus* required lactose molecule itself, because the equimolar mixture of glucose and galactose which constitute lactose molecule was inferior to lactose itself in the power to promote the development of *bidfius*-flora.

V. *Influences of Various Sugars for the Growth of L. bifidus in vitro.*

Although various sugars were transfused into the ilc. region for the purpose of increasing the sugar contents in that portion and promoting the development of *bifidus*-flora, no pure *bifidus*-flora could be obtained with any one sugar alone. Among the sugar employed, however, only lactose could accelerate the multiplication of this particular lactobacillus to certain extent. When pyruvic acid was administered with various sugars, lactose was proved to be extremely superior to other sugars in promoting the growth of the organism.

Thus, in the clinical experiments, lactose was most effective for the growth of this organism. Could this finding be true also *in vitro?* To answer this question, the influence of various sugars on the growth of *L. bifidus* have been studied *in vitro* using Petuely's medium (15).

1. Method.

a. Strains of *L. bifidus*: Four strains taken from the breast-fed infant.

b. Medium: Petuely's medium was used with addition of 0.3 percent pyruvic acid, with the pH adjusted to 6.4 with N/10 Na OH (Table 7).

c. Condition of Culture: Aerobic culture at 37° C for 72 hours.

d. Measurement of the Growth of *L. bifidus*: Growth was estimated by titration and expressed in ml. of N/10 Na OH required to neutralize the acid produced.

e. Sugars: Fructose, sucrose, maltose, glucose, galactose, and equimolar mixture of glucose and galactose were used instead of lactose in Petuely's medium.

Table 7. Petuely's Medium

Lactose	3.5 gm.
$(NH_4)_2 SO_4$	0.2 gm.
Cystin	0.02 gm.
$K_2 H PO_4$	0.25 gm.
$MgSO_4 \cdot 7H_2O$	12.5 mg.
$FeSO_4 \cdot 7H_2O$	0.6 mg.
$MnSO_4 \cdot 2H_2O$	0.4 mg.
NaCl	0.6 mg.
Ca-Pantothenate	20.0 γ
L-Ascorbic Acid	2.0 gm.
Biotin	0.2 γ
Tween 80	0.1 gm.

Add. water to 1,000 ml. pH 6.4

2. Results (Table 8).

As shown in Table 8, lactose had the most striking ability for the growth of *L. bifidus*, followed by galactose, glucose and equimolar mixture of them, in order. Sucrose and fructose had least ability. Then, lactose was the best one among the sugars utilized by *L. bifidus* even in such a simple medium. It has been suggested that the role of lactose did not only lie in supplying energy for growth, since the results of experiments *in vivo* and *in vitro* were not in parallel with certain sugars.

Table 8. Growth of *L. bifidus* by Various Sugars expressed in Term of Acidity produced.
(Petuely's medium plus 0.3% pyruvic acid, 37° C, 48 hours)

Sugars \ Strains	A	B	C	D
Lactose	5.9 ml.	5.2 ml.	4.6 ml.	6.9 ml.
Glucose	3.5	0.8	0	0.6
Galactose	4.0	4.3	1.4	4.8
Fructose	1.0	Tr.*	Tr.	0.5
Glu + Gal †	1.4	2.1	0	0.9
Maltose	0.7	Tr.	1.0	Tr.
Sucrose	Tr.	Tr.	Tr.	Tr.

* Tr. = Trace.
† Glu + Gal = equimolar mixture of glucose and galactose.

3. Conclusion.

The ability of various sugars to promote the multiplication of *L. bifidus in vitro* was compared, using Petuely's medium with addition of pyruvic acid, and lactose was confirmed to be superior to others, suggesting that not only it can supply the source of energy, but also it demonstrates an unique position for the growth of *L. bifidus*.

DISCUSSION

Apart from the significance of *bifidus*-flora on infant nutrition, numerous attempts to promote the development of *bifidus*-flora by modified artificial feeding have been reported. For example, Sittler (16), Adam (1) and Bessau (2) have presented the so-called "Bifidus diet". Gerstley (5), Malyoth (6), Düstelwald (17) and Frisell (18) have also reported that a certain *bifidus*-flora could be produced by adding β-lactose, stereoisomer of common α-lactose, to cow's milk formula. In our country, Aoki (3) and Takabayashi (19) under the direction of Professor T. Takuma have indicated that they were successful in promoting the development of *bidfius*-flora by adding lactose, malt soup extract, heated cereal or refined powder of soy-bean, while, Hirata (7) has definitely shown the development of *bifidus*-flora on the bottle-fed infant using so called ABC-milk fortified with β-lactose, that has been supported by Sagawa (20), Takahashi (21) and Ogawa (22).

Promotion of the growth of this particular microorganism by lactose has long been recognized by many investigators, but there arise a question what would be the biochemical mechanism for this explanation?

It has been generally assumed that lactose is poorly absorbed from the intestinal tract of infant, as Bessau had reported, and it can reach the lower bowel and give arise an influence on promoting the growth of *L. bifidus*. Aoki (3) has insisted that it was possible to promote the increase of *L. bifidus* in the intestinal flora by feeding poor absorptive sugars to infant. But, this explanation was melery a speculative one and the reliable evidence has never been presented up to now.

Fortunately, as it became possible to aspirate the intestinal contents or to transfuse any solution into the alimentary tract of infant at any level by Kobe-Tubing procedure, which has been devised in our laboratory, the experiments to re-evaluate the hypothesis were undertaken.

To summarize these results:

1. The action of lactose alone for promoting the multiplication of *L. bifidus*:

It should be reiterated once again that lactose is possessed of an action, when it is orally administered, which would be likely to promote the growth of *L. bifidus* in the lower alimentary canal, as accumulated evidences were conclusive. It has been also shown that lactose was the most effective sugar for the growth of the organism. Neverthless, it was found that the percentage of the organism in stool would be limited, not beyond 50 percent, even when fed on over 15L.

For the purpose of promoting the growth of *L. bifidus* in stool, the optimal concentration of lactose in artificial feeding was estimated to be 15 percent as total, which was equivalent to addition of 10.5 percent of lactose to the whole evaporated milk. It was of interest, that the sugar concentration of the ilc. contents in this formula was equal to that of breast-fed infant, with reference to the concentration of total sugar or fraction of lactose. But on this formula, the percentage of *bifidus* in stool was confined to about 60 percent at highest. In spite of increased concentrations of sugar to the extent of 20L or 25L, the effect on the growth of *L. bifidus* was little. Moreover, such degree of the percentage of the organism in stool was far from the *bifidus*-flora seen in the stool of the breast-fed infants even when the maximal percentage was attained.

Although lactose can exert an stronger effect for promoting the multiplication of *L. bifidus* than other sugars, the pure *bifidus*-flora could never be developed by administration of lactose alone. Therefore, it is reasonable to assume that the pure *bifidus*-flora of the breast-fed infant could be due to other factor(s) besides lactose.

On the other hand, basing upon the results of vast investigations on absorption of various sugars from the infant intestine, Matsui (23) in our laboratory concluded that added lactose might be so poorly absorbed from the intestine unlike lactose contained in human milk, and the concentrations of lactose in the ilc. contents could not be over 2.4 percent on an average, because of a self-regulating mechanism of sugar in the infant intestine, the excessive lactose being absorbed from the intestine and excreted in urine.

Therefore, an elucidation on the multiplication of *L. bifidus* in stool, which was generally accepted up to now, should be corrected.

2. On the reinforced action of lactose by pyruvic acid.

As referred, lactose has an extremely strong activity for promoting the growth of *L. bifidus*, within limited range, yet, the *bifidus*-flora as found in breast-fed infants could hardly be explained on the single action of lactose. An explanation based on other than lactose should be sought. An addition of pyruvic acid has been attempted as it was found to be effective in an aerobic culture of *L. bifidus in vitro*, since the substance, which was effective in an aerobic culture in *vitro*, should be presumably sufficient to support the growth also *in vivo*, because the intestinal content of infant was found to be not so anaerobic (24) as they were supposed to be.

As shown in chapter 3 and 4, the pure *bifidus*-flora, which was entirely similar to that of breast-fed infants, was obtained by simultaneous transfusion of pyruvic acid with lactose into the ilc. region of infants fed on basal formula, notwithstanding the failure to obtain pure *bifidus*-flora with lactose. Moreover, the character of stool in this case was almost similar to that of the breast-fed infant. This is the first experimental treal in which pure *bifidus*-flora was successfuly obtained on the bottle-fed infants.

However, explanation for this accelerated bacterial growth with combined administration has never been established up to now. György and Kuhn (25) have isolated from human milk the oligosaccharides named "Bifidus-Factor", which consisted of L-fucose, glucose, galactose, and N-acetyl-glucosamine and highly promoted the growth of *L. bifidus* var. *pennsylvanicus*, and proved the existence of these substances in higher quantities in human milk than in cow's milk.

But no relation between these oligosaccharides and pyruvic acid plus lactose was suggested. Kuhn (26) also isolated "Lactaminsäure-Lactose" from cow's colostral milk, which showed strong activity *in vitro*. It was of interest that "Lactaminsäure" had the structure consisted of pyruvic acid and N-acetyl-mannosamine (N-acetyl-glucosamine at first, was corrected later), and a possibility that pyruvic acid might be combined with N-acetyl-hexosamine and lactose in the infant intestine to form a substance resembled to " Lactaminsäure-lactose " has been suggested.

At any rate, it was remakable that lactose was extremely effective among the sugars tested, followed by fructose or sucrose, and equimolar mixture of glucose and galactose was far inferior to lactose in growth-promoting activity for *L. bifidus*, when combined with pyruvic acid. These facts might suggest that lactose did not only act as the energy source, but also the structure of 4-β-D-galactosido-glucose itself was necessary to exhibit the growth-promoting activity. Petuely (27) has presented that " Lactulose ", an artificial sugar, possessed *bifidus*-activity, and it was considered to be of interest that this substance had the structure of 4-β-D-galactosido-fructose, the similar glycosidic linkage as lactose.

Considering the next problem, even though pyruvic acid plus lactose was necessary to form anything with *bifidus*-activity, can we explain the difference of the intestinal flora between the breast- and bottle-fed infant by pyruvic acid? According to the studies in our laboratory, pyruvic acid or α-keto acids were constantly present in the ilc. contents of infants fed on various milks, but the concentrations of these acids did not show so much difference between the breast- and bottle-fed infants (28), as to be a cause of the difference of the intenstinal flora of these two kinds of feeding. It was found that the difference of the intestinal flora between the breast- and bottle-fed infants could not be explained even with pyruvic acid.

3. On the fermentation of various sugars by *L. bifidus in vitro.*

Influence of different kinds of sugar for the growth of *L. bifidus* were studied in the experiments of chapter 5, and lactose was best to be fermented by *L. bifidus in vitro.* But, judging the results, suitable care must be taken, since the experiments were performed only in the simple medium and the competitive action of *L. bifidus* and other intestinal bacteriae were not investigated. And yet, it has been demonstrated that lactose was not only effective as an energy source, but also the structure as diose itself was required, because lactose was provéd to be more effective than a mixture of monoses of its components.

As mentioned above, in summary, it can be considered that the activity of lactose in promoting the growth of *L. bifidus* was superior than other kinds of sugar both *in vivo* and *in vitro,* regardless of lactose alone or of the combined use with pyruvic acid, and also this activity was not only due to the source of energy, but also due to the β-glycosidic linkage in its structure.

Up to now, although many problems remain to be solved, a sufficient proof has been shown that the pure *bifidus*-flora would be unobtainable without lactose, signfying that the role of lactose is the unique one for the growth of the organism.

SUMMARY

With Kobe-Tubing procedure, the intestinal contents of the ileocaecal region of infants, who were fed with various kinds of formula, could be aspirated, and the

relation between the percentage of *L. bifidus* in stool and the concentration of sugar in the ileocaecal contents was studied. The effects of various sugars on promoting the development of *bifidus*-flora by transfusing into the ileocaecal region with or without combined use of pyruvic acid were tested, and the influence of sugars on growth of *L. bifidus* were compared *in vitro*.

It was discussed that lactose was not only acting as source of energy but also it provides an essential chemical structure of indispensable nature and possessed the unique position for the growth of *L. bifidus*.

ACKNOWLEDGEMENT

The author wishes to express his deep appreciation to Prof. Y. Hirata and Dr. T. Kondo for their helps and criticisms in carrying out this work. The results were reported briefly at the 61st Annual Meeting of the Japanese Society of Pediatrics in 1958, by Prof. Y. Hirata.

REFERENCES

1. Adam, A.: Jb. Kinderhkd., **52**, 38, 1900., Ibid., **61**, 687, 1905.
2. Bessau, G.: Mschr. Kinderhkd., **63**, 4, 1935., Ibid., **68**, 297, 1937., Dtsch. Med. Wschr., **64**, 397, 1938.
3. Aoki, F.: Chiba Igakkai Zasshi., **17**, 1853, 1939.
4. Skole, S. M.: Archiv. Pediatr., **50**, 395, 1933.
5. Gerstley, S. M. et al: Ibid., **52**, 552, 1935.
6. Malyoth, G. & Kirimlidis.: Klin. Wschr., **18**, 1240, 1939.
7. Hirata, Y.: Jap. J. Pediatr., **5**, 1, 1952., **6**, 30, 157, 237, 302, 520, 595, 665, 836, 1953., **7**, 196, 1954.
8. Adam, A.: Zschr. Kinderhkd., **31**, 308, 1922.
9. Stoye, W.: Ibid., **31**, 313, 1922.
10. Kleinschmidt. H.: Klin. Wschr., **14**, 257, 1935.
11. Hirata, Y.: Pediatrica Japonica, **1**, 67, 1958.
12. Roe, J. H.: J. Biol. Chem., **212**, 335, 1955.
13. Block, R. Durrum, E. & Zweig, G.: A Manual of Paperchromatography and Paper Electrophoresis. Acad. Press Inc., New York p. 127, 1955.
14. Tamaki, T.: Kobe J. Med. Sci. (in press)
15. Petuely, F.: Zschr. Kinderhkd., **78**, 28, 1956.
16. Sittler, P.: Cbl. Bakt., I, **47**, 14, 1908.
17. Düsterwald, W. et al: Med. Klin., **45**, 1143, 1950.
18. Frisell. E.: Act. Pediatr., **80**, 1, 1951.
19. Takabayashi, T.: Act. Pediatr. Jap., **48**, 1078, 1942., **49**, 11, 83, 155, 249, 341, 485, 605, 1943.
20. Sagawa, I.: Act. Pediatr. Jap., **57**, 534, 1953.
21. Takahashi, T.: Ibid., **59**, 783, 791, 1955.
22. Ogawa, I.: Ibid., **62**, 106, 1958.
23. Matsui, M.: Kobe J. Med. Sci. (in press)
24. Tamaki, T.: Kobe J. Med. Sci. (in press)
25. György, P., Kuhn, R.,Rose C. S. & Zilliken, F.: Arch. Biochem. & Biophys., **48**, 209, 1954.
26. Kuhn, R.: Angew. Chem., **69**, 23, 1957.
27. Petuely, F.: Zschr. Kinderhkd., **79**, 174, 1957.
28. Kokubu, H.: Kobe J. Med. Sci. (in press)

Fig. 1. Smears of the stool of the infant fed on the basal formula, before simultaneous transfusion of lactose and pyruvic acid. Gram-stain.
Magnification : 900×

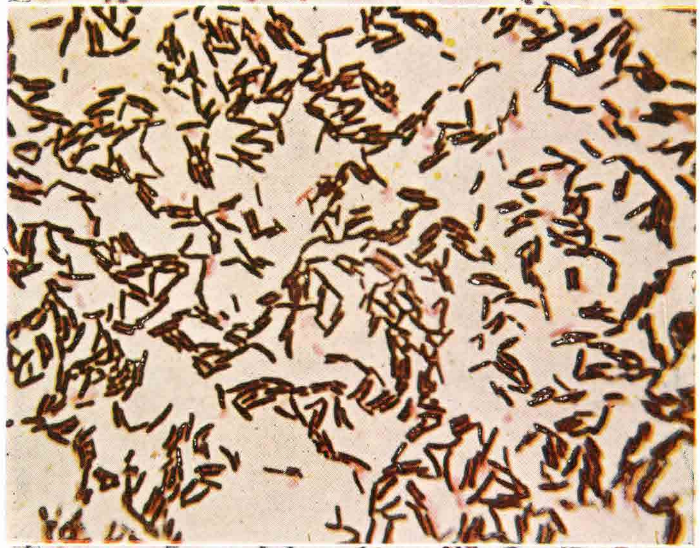

Fig. 2. Smears of the stool of the infant fed on the basal formula, after simultaneous of lactose and pyruvic acid. Gram-stain.
Manification : 900×

Fig. 3. Smears of the stool of the infant fed on human milk. Gram-stain.
Magnification : 900×