Enhancement of intestinal hydrolysis of lactose by microbial β-galactosidase (*EC* 3.2.1.23) of kefir

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The effect of microbial β-galactosidase (*EC* 3.2.1.23) activity on intestinal lactose digestion was estimated directly by following post-prandial venous plasma galactose concentrations. To avoid superimposing effects of free galactose, as with yogurt, fresh or heat-treated suspensions of mechanically disintegrated kefir grains in kefir, containing lactose but no free galactose, were fed to ten Göttingen minipigs. Each meal contained 101·1 (SEM 0·1) mmol lactose in kefir supplemented by either native or heat-treated kefir grains corresponding to a mean β-galactosidase activity of either 72 (SEM 8) U or zero. Feeding kefir with β-galactosidase activity resulted in a 30% enhancement of the mean post-prandial plasma galactose peak concentration from 33 (SEM 7) to 43 (SEM 12) μmol/l (*n* 10), as well as in 23% greater mean areas under the galactose-response curves (8·1 (SEM 1·5) *v.* 6·6 (SEM 1·2) mmol/min per l) if compared with kefir with heat-treated grains. Both differences were significant ($P < 0·05$; paired Wilcoxon test by ranks). There was no induction of intestinal β-galactosidase (*EC* 3.2.1.108) activity or intestinal lactose-hydrolysing bacteria by lactose feeding. These results give direct evidence of an enhanced lactose digestion and absorption in native fermented milk products due to the microbial β-galactosidase activity.

β-Galactosidase: Lactose digestion: Plasma galactose

Depending on ethnic origin, from 15 to 80% of adult populations have low activities of β-galactosidase (*EC* 3.2.1.108) in the intestinal mucosa (Dahlqvist, 1983). In cases of low mucosal β-galactosidase activity there may be signs of intolerance caused by the osmotic effect of lactose reaching the distal part of the intestine, and by the volatile end-products of bacterial fermentation of this undigested lactose, i.e. volatile organic acids, carbon dioxide, methane and hydrogen (Auricchio *et al.* 1963; Dahlqvist *et al.* 1963).

A number of laboratories have reported evidence that intestinal hydrolysis of lactose can be enhanced if it is consumed together with lactobacilli in a fermented milk product like yogurt (Gallagher *et al.* 1974; Gilliland & Kim, 1984; Kolars *et al.* 1984; Savaiano *et al.* 1984; Martini *et al.* 1987*a*; Dewit *et al.* 1988; Lerebours *et al.* 1989; Onwulata *et al.* 1989; Marteau *et al.* 1990). This enhancement was attributed to the bacterial β-galactosidase (*EC* 3.2.1.23) of the fermented food and the activity of this enzyme was shown to occur in duodenal aspirates following yogurt consumption (Goodenough & Kleyn, 1976; Kolars *et al.* 1984; Conway *et al.* 1987; Pochart *et al.* 1989).

This evidence about the 'autodigestive' activity of yogurt was based on H_2 exhalation, a method dependent on the assumption that maldigested lactose causes more H_2 formation in the lower part of the intestine (McGill, 1983).

More direct evidence substantiating such an enhancement of lactose digestion would consist of a rise in post-prandial plasma galactose concentrations reflecting a more rapid

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formation of this product by bacterial β-galactosidase. However, several attempts to prove such a rise in post-prandial galactose have been unsuccessful (Goodenough & Kleyn, 1976; Schaafsma *et al.* 1988). This was probably due to the fact that in these studies yogurt was used as the test meal. Yogurt, however, contains considerable concentrations of free galactose (Alm, 1982) preformed by the yogurt culture, and this galactose probably contributes so much to the plasma levels, that a stimulatory effect of bacterial β-galactosidase is not distinguishable.

The present experiments use kefir instead of yogurt, thus exploiting the fact that the former contains β-galactosidase activity in the grains but no free galactose (Alm, 1982).

Kefir is a cultured milk product traditionally prepared from cows' milk by inoculation of so-called kefir grains, small cauliflower-like particles consisting of several kinds of lactobacilli and of lactose-fermenting and non-lactose-fermenting yeasts, embedded in an elastic polysaccharide matrix of bacterial origin. The β-galactosidase activity of the kefir grains results mainly from the lactobacilli (e.g. *Lactobacillus brevis*, *Lactobacillus kefyr*, *Lactobacillus buchneri*), whereas lactose-fermenting, ethanol- and CO_2-producing yeasts (e.g. *Candida kefyr*) show a lower activity. Other yeasts (e.g. *Saccharomyces* spp.) have no β-galactosidase. These lactose-non-fermenting yeasts in the kefir grains use lactic acid or galactose as the only carbon source; the latter activity explains the lack of free galactose in kefir (Engel, 1984; Hirota, 1987).

MATERIALS AND METHODS

Animals

The experiments were performed with ten adult female Göttingen minipigs (mean weight 29 kg) housed in metabolism cages and fitted with permanent catheters in the external jugular vein. Total intestinal β-galactosidase activity of the pigs was about 170 U (lactose as the substrate); the mean specific activity in the proximal jejunum was 11 (SEM 2) U/g protein (*n* 4). This activity was estimated at ten equidistant points in the small intestine of four pigs. Segments of the intestine were homogenized, incubated with lactose and the galactose formed was estimated enzymically (Kidder & Manners, 1980). No further differentiation was made between different kinds of intestinal β-galactosidase (lactase).

On days when the pigs did not receive a kefir meal they were kept on a semi-synthetic balanced diet (diet C 22; Pfeuffer *et al.* 1988) but with 150 instead of 220 g casein/kg).

Kefir

Kefir was a gift of Müller, Aretsried, Germany. Kefir grains were obtained from Laboratory Wiesby, Niebüll, Germany and contained *L. kefyr*, *Lactococcus lactis* (subsp. lactis/cremoris), the lactose- and galactose-fermenting yeast *Candida kefyr* and the lactose non-fermenting, galactose-metabolizing yeast *Saccharomyces unisporus* (estimated by Laboratory Wiesby, Quality Control Department). Kefir grains (10 g per animal) and meal were disintegrated in 20 ml saline (9 g sodium chloride/l) using a household mixer (2 min at 20°). The suspension was added directly or after heat treatment (10 min at 100°) to 1 litre kefir to reach final concentrations of 100 mmol lactose/l and 72 U β-galactosidase/l; the latter was estimated with lactose as the substrate.

These activities are similar to those measured in commercial yogurts. Kefir β-galactosidase activity was estimated at its optimum pH of 6; it was totally destroyed at pH 2.

Experimental design

The experiments lasted 2 weeks. The pigs were randomly divided into two groups of five animals each. On 3 d of each experimental week they were given, after a 24 h fast, 1 litre kefir containing either fresh or heated kefir grains. Group 1 started with heat-treated grains

in the first week, followed by fresh grains in the second week, whereas group 2 received kefir with fresh grains in the first and with heated grains in the second week. The time-interval between adding the disintegrated grains and feeding the test meal was less than 30 min.

Blood was withdrawn from the external jugular vein through the catheter immediately before and 30, 60, 90, 120, 180, 240, 300 and 420 min after feeding. Fluoridized blood samples were kept on ice and centrifuged as soon as possible for 10 min at 3000 *g*. The plasma and portions of each test meal were stored at −70° until analysis.

Analytical methods

The lactose, galactose and glucose contents of the kefir were determined in the clear filtrate obtained after deproteinization with Carrez's reagent using common enzymic methods (Boehringer Mannheim, 1986).

The β-galactosidase activity of the kefir grains was assayed using lactose (Dahlqvist, 1984*b*) or *o*-Nitrophenyl galactoside (o-NPG) (Hestrin *et al.* 1955) as the substrates. The grains were prepared according to Hirota (1987). This procedure was optimized with respect to maximum activity. Optimum conditions were: freezing (−70°) and thawing, followed by disintegration of the grains with saline in a household food mixer for 2 min and, thereafter, for 15 min at 1500 rev./min using a Potter-Elvehjem homogenizer. Sonication of the grains was unsuccessful due to the elastic matrix of the grains.

The buffering capacity of kefir compared with yogurt and milk was estimated by titration of 50 ml milk or milk product with 1·0 M-hydrochloric acid and 1·0 M-sodium hydroxide, to change the pH from 4·1 to 2·0 and from 2·0 to 7.

The following procedure was followed to prove whether incubation of kefir with disintegrated grains leads to the formation of free galactose during the period between preparing and feeding the test meal: 1 ml of a suspension of 1 g fresh kefir grains disintegrated mechanically in 10 ml distilled water was added to 10 g kefir. After an incubation period of 1 h at 37° the suspension and the kefir without grains were assayed enzymically for galactose, glucose and lactose (Boehringer Mannheim, 1986).

Blood galactose (Beutler, 1984) and glucose (Kunst *et al.* 1984) were estimated enzymically in plasma samples neutralized after deproteinization with perchloric acid. Individual peak galactose and glucose increments were taken as the maximum increase in plasma concentration from the fasting level irrespective of the time at which it occurred. Integrated incremental responses (areas under the curve) were calculated using a trapezoid method.

In order to examine whether prolonged lactose feeding increased β-galactosidase activity in mucosal cells or stimulated the growth of lactose-hydrolysing intestinal bacteria, both resulting in increased intestinal lactose hydrolysis and digestion, the experimental groups 1 and 2 (see pp. 67–68) were analysed separately. The reasoning for this was based on the following: if there is any stimulation of lactose-hydrolysing intestinal bacteria or the intrinsic β-galactosidase by previously feeding lactose, then total β-galactosidase activity in the intestine of the pigs is composed as follows (where I is intrinsic intestinal activity, M is microbial activity from the kefir and S is lactose-stimulated activity): group 1, first week (heated grains), I; group 1, second week (fresh grains), I + M + S; group 2, first week (fresh grains), I + M; group 2, second week (heated grains), I + S.

In group 1 the difference in total β-galactosidase activity between the first and the second week is: (I + M + S) − I = M + S, in group 2 this difference is only (I + M) − (I + S) = M − S. It is obvious from this scheme that the difference in plasma galactose between the first and the second week, which depends on total β-galactosidase activity in the intestine, would be greater in group 1 than in group 2 in the case of a stimulation of intestinal enzyme activity

by previous lactose feeding. Without stimulation (S = 0) this difference should be the same (M) in the two groups.

Statistics

Mean values with their standard errors are given throughout. Statistical analysis was done by paired Wilcoxon test by ranks.

RESULTS

Carbohydrates and β-galactosidase activity in the diets

Pigs were given a total β-galactosidase activity of 72 (SEM 8) U or less than 0·1 U respectively when a fresh or heated suspension of kefir grains was added to the kefir (Table 1). These values were obtained with lactose as the substrate. When assayed with o-NPG enzyme activities were 8·3 times higher.

Lactose in the test meal amounted to 101·1 (SEM 0·1) mmol. Neither free galactose nor glucose were detectable (Table 1).

Suspending kefir grains in saline did not disturb their galactose-metabolizing activity. Incubation with mechanically disintegrated grains did not result in the formation of free galactose or glucose in the kefir within 1 h (Table 2), a period twice as long as that between preparing and feeding the kefir grain suspension. During this period the lactose concentration in the kefir decreased by 4·8 mmol/l. This corresponded well with the added β-galactosidase activity of 73 U/l.

This model experiment in vitro precluded the possibility that more than negligible amounts of free galactose or glucose were formed in the test meal before it reached the duodenum.

Table 3 shows that the buffering capacities of kefir and yogurt, being similar, exceeded that of milk.

Plasma galactose

When the pigs were fed on kefir with heated grains, the average plasma galactose concentration rose from undetectable levels to 33 (SEM 7) μmol/l within 60 min. This was presumably due to the intrinsic β-galactosidase activity of the pigs. Feeding kefir with fresh grains led to a greater increase (by 30%, $P < 0·05$) in plasma galactose. The average maximum concentration of 43 (SEM 12) μmol/l was reached 90 min post prandially (Fig. 1).

Higher maximum plasma galactose concentrations after kefir with fresh grains compared with heated grains were observed in nine of ten pigs, the average of all individual differences of 10 (SEM 6) μmol/l being significantly different from zero ($P < 0·05$).

Table 4 shows that mean areas under the time *v.* concentration curves were significantly higher (by 23%) following fresh kefir grains instead of heated grains. Larger areas were observed with fresh grains in nine of ten animals.

Plasma glucose

The mean values for plasma glucose concentration increased from the fasting level of 4·80 (SEM 0·20) mmol/l to a maximum of 5·50 (SEM 0·20) or 5·45 (SEM 0·02) mmol/l respectively within 180 min following a test meal with fresh or heated grains (Fig. 2). The glucose peak was broader and later compared with that of galactose.

The areas under the curves following consumption of fresh or heated kefir grains were not significantly different (Table 4). However, during the first 90 min post prandially, pigs fed on kefir with fresh grains showed a tendency towards higher plasma glucose concentrations at each time-interval compared with pigs fed on heated grains (Fig. 2). The corresponding mean values of the areas under the response curves were 11·6 (SEM 8·7) mmol/min per l after fresh grains and 4·5 (SEM 6·1) mmol/min per l after heat-treated grains. This difference did not reach statistical significance ($P > 0·05$).

Table 1. *Biochemical composition of the test meals*

(Mean values with their standard errors for six experimental days; measured immediately after incubation with kefir grains when 1 litre kefir was fed to the pigs)

	Kefir* with fresh grains (*n* 6)		Kefir with heated grains (*n* 6)	
	Mean	SEM	Mean	SEM
Lactose (mmol/l)	101·1	0·1	101·1	0·1
Glucose (mmol/l)	nd	—	nd	—
Galactose (mmol/l)	nd	—	nd	—
β-Galactosidase (*EC* 3.2.1.23; U/l)†	72	8	nd	—
pH	4·4	—	4·4	—

nd, not detectable.

* Cultured milk product traditionally prepared from cow's milk by inoculation of so-called kefir grains; for details, see p. 68.

† Estimated with lactose as the substrate.

Table 2. *Influence of 1 h incubation* with fresh kefir† grains on lactose, glucose and galactose concentration in kefir*

	Kefir without grains (*n* 6)	Kefir incubated for 1 h (*n* 6)
Lactose (mmol/l)	103·2	98·4
Glucose (mmol/l)	nd	nd
Galactose (mmol/l)	nd	nd
β-Galactosidase (*EC* 3.2.1.23; U/l)‡	nd	73
pH	4·9	4·9

nd, not detectable.

* Kefir (10 ml) was incubated at 37° for 1 h with a suspension of 0·1 g disintegrated kefir grains in 1 ml distilled water.

† Cultured milk product traditionally prepared from cow's milk by inoculation of so-called kefir grains; for details, see p. 68.

‡ Estimated at pH 6 with lactose as substrate.

Table 3. *Buffering capacity of kefir* and other dairy products†*

	Kefir	Yogurt	Milk
pH 4·1 → 2·0 (ml 1 M-HCl)	5·8	6·4	3·3
pH 2·0 → 7·0 (ml 1 M-NaOH)	10·1	11·5	6·4

* Cultured milk product traditionally prepared from cow's milk by inoculation of so-called kefir grains; for details, see p. 68.

† Hydrochloric acid (1 M) or 1 M-sodium hydroxide required to shift the pH, as indicated.

Stimulatory effect of lactose

Table 5 shows the results of the separate analysis of the two experimental groups, i.e. pigs fed on heated grains in the first week (group 1) *v.* pigs that started with fresh grains (group 2). The differences in total post-prandial galactose response between β-galactosidase-

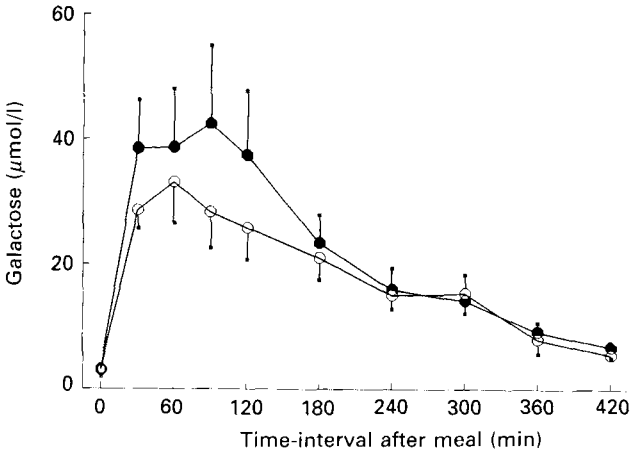

Fig. 1. Post-prandial plasma galactose concentrations in pigs fed on a fresh (●) or heat-treated (○) suspension of mechanically disintegrated kefir grains in 1 litre kefir (cultured milk product traditionally prepared from cow's milk by inoculation of so-called kefir grains; for details, see p. 68). Each animal received a total of 100 mmol lactose and either 600 (fresh grains) or less than 0·1 U (heated grains) β-galactosidase (*EC* 3.2.1.23). Samples were taken via a jugular catheter at time-intervals indicated. Values are means with their standard errors represented by vertical bars for ten pigs.

Table 4. *Post-prandial plasma glucose and galactose responses* in pigs fed on kefir† with fresh or heated kefir grains*

(Mean values with their standard errors)

	Kefir with heated grains (*n* 10)		Kefir with fresh grains (*n* 10)		Statistical significance of difference‡:
	Mean	SEM	Mean	SEM	*P*
Galactose (mmol/min per l)	6·6	1·2	8·1	1·5	< 0·05
Glucose (mmol/min per l)	157	36	163	29	NS

NS, not significant.

* Areas under the response curves (Figs 1 and 2).

† Cultured milk product traditionally prepared from cow's milk by inoculation of so-called kefir grains; for details, see p. 68.

‡ Paired Wilcoxon test by ranks.

containing and β-galactosidase-free test meals following lactose feeding were higher in pigs which started with fresh kefir grains (group 2). As explained previously, the contrary would be expected in the case of a lactose-induced stimulation of lactose-hydrolysing intestinal bacteria (see p. 69).

DISCUSSION

The present investigation shows unequivocally that the consumption of fermented milk products with microbial β-galactosidase activity causes higher post-prandial plasma galactose concentrations compared with heat-treated fermented milk products without β-galactosidase. Previous investigations in rats did not result in differences in post-prandial plasma galactose between fresh or pasteurized yogurt (Schaafsma *et al.* 1988). Other investigators reported differences which correlated primarily with the amount of free galactose in the test meal and a preceding lactose feeding period, precluding an unequivocal conclusion about an effect of microbial β-galactosidase (Goodenough & Kleyn, 1976).

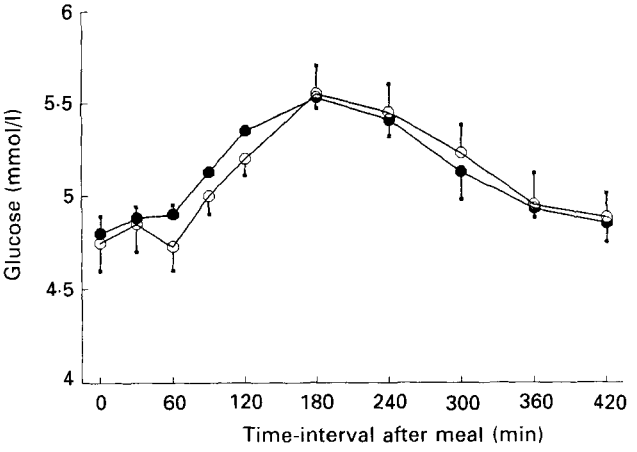

Fig. 2. Post-prandial plasma glucose concentrations in pigs fed on a fresh (●) or heat-treated (○) suspension of mechanically disintegrated kefir grains in 1 litre kefir (cultured milk product traditionally prepared from cow's milk by inoculation of so-called kefir grains; for details, see p. 68). Each animal received a total of 100 mmol lactose and either 600 (fresh grains) or less than 0·1 U (heated grains) β-galactosidase (*EC* 3.2.1.23). Samples were taken via a jugular catheter at time intervals indicated. Values are means with their standard errors represented by vertical bars for ten pigs.

Table 5. *Influence of the feeding sequence on the difference between post-prandial plasma galactose responses* in pigs after kefir† with heated or fresh grains*

(Mean values with their standard errors)

	Group 1 (*n* 5)		Group 2 (*n* 5)		Statistical significance of difference‡: *P*
Week ...	1	2	1	2	
Treatment of kefir grains ...	Heated	Fresh	Fresh	Heated	
Change in galactose area (mmol/min per l): Mean	1·0		2·5		NS
SEM	0·7		1·0		—

NS, not significant.

* Mean values for the differences (unheated minus heated) between the areas under the galactose response curves after heated and fresh grains. Only the first of the three experimental days of the first week and the last experimental day of the second week have been taken into account.

† Cultured milk product traditionally prepared from cow's milk by inoculation of so-called kefir grains; for details, see p. 68.

‡ Paired Wilcoxon test by ranks.

In the present study both peak galactose concentration and the area under the galactose response curve were significantly increased when a suspension of mechanically disintegrated fresh kefir grains in kefir was fed to Göttingen minipigs instead of kefir with heat-treated grains. Since the kefir contained no free galactose it is obvious that the rise in plasma galactose resulted from increased intestinal lactose hydrolysis.

Only post-prandial plasma galactose concentrations, not glucose, were significantly enhanced when kefir with β-galactosidase activity was fed. Changes due to lactose digestion were small compared with the fasting glucose level and overshadowed by the general post-prandial rise in plasma glucose concentrations. However, in the first 90 min post-prandially, mean plasma glucose concentrations tended to be higher in pigs fed on kefir with fresh grains though not reaching statistical significance.

The feeding of kefir with fresh instead of heated grains enhanced intestinal lactose

hydrolysis in the pigs, as estimated from areas under the galactose response curves, by 23%. This value seems not to be very high compared with the results of breath H_2 measurements, where consumption of unpasteurized yogurt decreased H_2 exhalation by 57–75% in lactose-intolerant subjects (Gilliland & Kim, 1984; Kolars *et al.* 1984; Savaiano *et al.* 1984; Martini *et al.* 1987*a*; Dewit *et al.* 1988; Lerebours *et al.* 1989). However, the total intrinsic intestinal β-galactosidase activity of the pigs was about 170 U, the specific activity in the proximal jejunum was about 11 U/g protein, whereas the activity estimated in human lactose malabsorbers is less than 5 U/g protein (Dahlqvist 1984*a*). Therefore, the percentage enhancement of lactose hydrolysis is less in pigs than in lactase-deficient subjects. Each test meal contained approximately 42% of intestinal β-galactosidase activity of the pigs. Therefore, an increase in lactose hydrolysis of 23% would indicate that almost 55% of the β-galactosidase fed was still active in the intestine.

Microbial β-galactosidase is rapidly and irreversibly inactivated below pH 2 but relatively stable at values above 4 (Conway *et al.* 1987; Martini *et al.* 1987*b*). So the pH of gastric juice and, therefore, the buffering capacity of the ingested milk product may be a determinant factor for the survival of β-galactosidase activity passing through the acid milieu of the stomach. Kefir proved to be an excellent buffer like yogurt and this may partly explain why kefir with grains enhances intestinal lactose hydrolysis as effectively as yogurt.

In the present paper we have not differentiated between the β-galactosidase activity of the lactobacilli and of lactose-fermenting yeasts (e.g. *Candida kefyr*) of the kefir fed. There is evidence that oral treatment of human volunteers and rats with *Saccaromyces boulardii*, a yeast not contained in kefir, enhances intestinal β-galactosidase activity (Buts *et al.* 1986). In kefir only a minor part of β-galactosidase results from yeast, therefore the effects described herein have to be attributed mainly to the bacterial enzyme activity.

In addition to the direct action of ingested microbial β-galactosidase, there may be effects of long-term feeding of lactose or microbial β-galactosidase, which are responsible for enhanced intestinal lactose hydrolysis. Such mechanisms are: a shift from non-lactose-fermenting to lactose-fermenting bacteria in the intestinal flora after prolonged ingestion of lactose (Hill, 1983), stimulation of the intrinsic mucosal β-galactosidase (Besnier *et al.* 1983) or adherence of microbial β-galactosidase from the fermented milk product to mucosal cells (Garvie *et al.* 1984). The present study gave no evidence for a stimulatory effect of repeated feeding of kefir with β-galactosidase activity on intestinal lactose digestion in the pig. This is in accordance with investigations in lactase-deficient individuals, where ingestion of fresh or pasteurized yogurt for 1 week did not increase intestinal β-galactosidase activity (Lerebours *et al.* 1989).

It is a task for future research to extend these results obtained with a 'model food', kefir with grains, to a more common fermented food, e.g. yogurt. In summary, the results presented herein provide further strong and direct evidence that the consumption of fermented instead of fresh dairy products is a rational approach to avoid symptoms of lactose malabsorption in lactose-deficient subjects.

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