THE INFLUENCE OF REGULAR CONSUMPTION OF KEFIR BEVERAGE ON THE INCIDENCE OF ENTEROCOCCUS FAECALIS IN THE HUMAN STOOL

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A b s t r a c t

The subject of this study was to test the influence of regular consumption of kefir beverage (Danone company) comprising probiotic bacterial strains (*Bifidobacterium animalis* 10^8 CFU/g^{-1}, *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus sp., Leuconostoc mesenteroides...*), inulin, and yeasts (*Debaryomyces hansenii, Pichia fermentas, Torulopsis holmii*) on the incidence of *Enterococcus faecalis* in the human stool.

Five hundred women (age 18–55), who had suffered from various forms of indigestion, were selected by questionnaires. We then selected from this group 50 volunteers who consumed a 200 ml kefir beverage regularly, each evening, after dinner. Their stool samples were taken before the research period started, and after two weeks of kefir beverage consumption. The women also filled in the questionnaire about their subjective feeling (alimentary function) after 14 days of kefir beverage consumption.

The microbiological cultivation was carried out on a special medium Slanetz Bartley – pick-up *Enterococcus faecalis* (M 612 HiMEDIA). The average number of *Enterococcus faecalis* per 1 g of stool was $4.48.10^8$ CFU at the beginning of the study (median $5.25.10^7$ CFU, SD $1.18.10^9$ CFU), while after 14 days of regular daily consumption of the kefir beverage the value determined was $3.32.10^7$ CFU (median $2.65.10^6$ CFU, SD $8.56.10^7$ CFU). The sign test, resultant value u = 4.808326 (critical value 2.58 for statistical significance p = 0.01) showed that the difference in the counts of *Enterococcus faecalis* in the stool of individuals intervened prior and subsequent to the consumption of kefir beverage was statistically significant, $p < 0.01$. The counts of *Enterococcus faecalis* in the stool of individuals after kefir beverage consumption were significantly lower. A subjective evaluation of the women in the questionnaire showed that 93.5 % noticed, after a regular 14 days' consumption of 200 ml kefir beverage, improved alimentary function in comparison with the period before the study ($p < 0.001$).

INTRODUCTION

Good nutrition plays a significant role in the regulation and proper functioning of the human intestine. Besides regular eating and sufficient liquid intake, it is also necessary to maintain the proportion of the so-called functional foods in the diet

(*1*). Functional foods are natural or formulated foods that enhance physiological performance, prevent or treat diseases (*2*).

Kefir beverages, among other forms of sustenance, belong among the above-mentioned functional foods. Kefir beverage is a fermented dairy beverage containing a very diverse array of microorganisms and yeasts (*3*). A range of them have probiotic effects which are beneficial to human health (*4, 5*). These are mainly some of the bacterial strains of *Lactobacillus, Streptococcus*, *Bifidobacterium, Lactococcus or Leuconostoc* (*6*), and also yeasts.

The aim of this study was to investigate the influence of regular consumption of kefir beverage comprising probiotic bacterial strains (*Bifidobacterium animalis, Lactococcus lactis, Streptococcus thermophilus, Lactobacillus sp., Leuconostoc mesenteroides*...), inulin, and yeasts (*Debaryomyces hansenii, Pichia fermentas, Torulopsis holmii*) on the incidence of *Enterococcus faecalis* in the human stool.

Significant results were obtained in a study which was monitoring the effect of the *Lactococcus lactis* strain on the composition of the intestinal microbiota of human flora-associated rats. The number of *Bifidobacterium* cells in faecal samples significantly increased, and the number of Enterococci and Streptococci in duodenum, ileum, caecum, and colon decreased (*7*).

The probiotic influence on enteric pathogenic bacteria was also established in a study carried out by *Jacobsen and Nielsen* (*8*) in selected strains of *Lactobacillus* spp.

In the group of Bifidobacteria, there is a range that has recently been used in dairy products. The most frequently examined strain seems to be the *Bifidobacterium animalis* DN 173 010. It has an exceptional capability of overcoming the acidic environment in the stomach (*9, 10*) and it passes in comparatively large quantities through the small intestine into the large intestine (*11*), and even after passing through the alimentary tract it can be found in large live quantities in human stool samples (*12*). Live bacteria of *Bifidobacterium animalis* DN 173 010 can also significantly reduce the time of the passage through the intestines (*13, 14, 15*).

Yeasts have the ability to metabolise organic acids, galactose and glucose derived from bacterial metabolism of milk lactose, free fatty acids or free amino acids present in dairy products. This results in a decrease in acidity. Thus yeasts assure better survival of the probiotic bacteria in bio-yoghurt and kefir and their further beneficial function in the human gastrointestinal tract (*16*). Some yeasts are resistant to acidic conditions and have the capacity to adhere to human enterocyte-like Caco-2 cells (*17, 18*).

Inulin was another interesting ingredient of our kefir beverage. It is a fructooligosaccharide, an undigested substance, which passes from the small intestine into the large intestine. It is a substance necessary for the bacteria that are beneficial to human health. It supports the development and reproduction of these bacteria (19, 20). In a study carried out in Germany, inulin was administered to humans for 64 days in dosages of 34g/day, which significantly increased the number of Bifidobacteria in their stool samples (*21*).

MATERIALS AND METHODS

The research ran for 2 months in the Department of Preventive Medicine. Via questionnaires we selected, in cooperation with the TNS AISA agency, 500 female participants who had problems with their digestion (constipation, flatulence, feeling heavy). The participants were approached by trained questioners with whom they filled in questionnaires concerning the healthiness of their lifestyle, and focusing on the proper functioning of the alimentary tract.

From the above-mentioned group 50 volunteers were then selected, between 18 and 55 years of age, who were willing to deliver stool samples and, equally importantly, had not consumed any probiotic dairy product in the previous three weeks and had not been on any specific diet. These women were instructed to drink a 200 ml kefir beverage each evening after their evening meal for a fortnight. In the course of the study they were not permitted to consume any other probiotic dairy beverage.

The said kefir beverage contained several probiotic bacterial strains (*Bifidobacterium animalis* 10^8 CFU/g^{-1} – by Danone company, *Lactococcus lactis*, *Leuconostoc mesenteroides, Streptococcus thermophilus, and Lactobacillus* spp.), prebiotic inulin, and yeasts (*Debaryomyces hansenii, Candida lambica, and Torulopsis holmii*). The above-listed bacterial strains and yeasts are only the most significant representatives of the kefir beverage. Except *Bifidobacterium animalis* we did not know exactly the counts of other bacteria in kefir beverage.

The selected sample of women brought their stool samples to the Department of Preventive Medicine to be examined before the beginning of the said course of kefir beverage consumption. The women had taken the stool samples at home, after being thoroughly instructed on how to do it by a team of examiners. A sterile set consisting of a plastic stick (in the shape of a hockey stick) and a Petri dish was provided; the women were asked to place a sample of approximately 3x3 cm in size in the dish for sampling. Part of the set was also detailed instructions for stool sampling. The women were to keep the samples packed in a separate compartment of their refrigerator (5 ^{0}C). They would then bring the samples to the Department of Preventive Medicine in a thermo-bag. The same procedure followed after the fourteenth day of consuming the above-described product.

An analysis of the samples was carried out together with the microbiological cultivation of both the stool samples taken before and after the consumption of the kefir beverage. An important part of the study was verification of the methods and estimation of the required numbers of dilutions, which was practically tested on a third of the samples taken a week before the research started.

The Slanetz Bartley medium (M 612 HiMEDIA) was used as a selective medium for the cultivation of *Enterococcus faecalis*. Five grammes from each stool sample were placed in a sterile flask containing 45 ml of saline solution. The flasks were shaken thoroughly on a shaking machine for 30 minutes to mix the stool and the saline properly. Then a dilution was carried out using an automatic pipette (1ml). It was necessary to determine the total number of *Enterococcus faecalis*, making up the dilution to 10^{-5}. The samples, after their proper dilution, were added to the Slanetz Bartley medium. Always, three dilutions were tested (in double) according to the results obtained from the test samples. 0.2 ml of the suspension of the diluted stool sample was added on the Petri dish. Petri dishes with the Slanetz Bartley medium were put into a heating unit, after letting the suspension dry for a short time, and then left to cultivate for 48 hours at a temperature of 37 ^{0}C.

After the cultivation was complete, the colonies of *Enterococcus faecalis* grown on the Slanetz Bartley medium were quantitatively counted.

Before the start of the second phase of the assessment of the stool samples (after the regular kefir beverage consumption), another test cultivation was carried out on a third of the stool samples, with the aim of finding out the dilution for the second set of samples, and to prevent unnecessary wastage of material and time due to unnecessarily repeated cultivations. Then the second phase of assessments was carried out.

We have statistically processed the data acquired using the EPI INFO 6 and our own programme in Python Programming Language. In our programme, the counts of positive and negative results of the difference (bacteria count before the intervention – bacteria count after the intervention) were enumerated. Decadic logarithms were taken of the absolute values of the resultant differences and also

of the corresponding minuends and subtrahends. The input values were plotted in a two-dimensional graph. For the assessment of statistical significance we chose the Sign Test for Paired Data, which tests the hypothesis that the median of the differences in the pairs is zero (*22*). The result of the latter was converted to the p value.

The women also filled in questionnaires about their subjective feeling (alimentary function) after kefir beverage consumption.

RESULTS

The total counts of *Enterococcus faecalis* in the stool (CFU/g^{-1}) of the monitored individuals prior and subsequent to intervention found via cultivation on the Slanetz Bartley medium are given in *Table 1*. The counts of *Enterococcus faecalis* in the stool of monitored individuals prior and subsequent to intervention found by cultivation on the Slanetz Bartley medium were expressed in the exponential form as pairs of numbers.

The average number of *Enterococcus faecalis* per 1 g of stool was $4.48.10^8$ CFU at the beginning of the study (median $5.25.10^7$ CFU, SD $1.18.10^9$ CFU), while after 14 days of regular daily consumption of the kefir beverage the value determined was $3.32.10^7$ CFU (median $2.65.10^6$ CFU, SD $8.56.10^7$ CFU).

The distribution of the logarithms of positive and negative differences on the numerical axis is given in *Fig. 1*. There are significant differences from the casual values.

The sign test, resultant value u = 4.808326 (critical value 2.58 for statistical significance p = 0.01), showed unambiguously that the difference in the counts of *Enterococcus faecalis* in the stool of intervened individuals prior and subsequent to a 14-day consumption of kefir beverage was statistically significant, $p < 0.01$. The counts of *Enterococcus faecalis* in the stool of individuals after kefir beverage consumption were significantly lower.

In total, 93.5 % of the participants declared in the questionnaire their subjective feeling that after a regular 14 days' consumption of the kefir beverage they noticed an improved alimentary function in comparison with the period before the study. Negative change was noticed by 6 % of the respondents; the rest of the sample did not mention any change. The results were statistically significant ($p < 0.001$).

DISCUSSION

The method of selecting participants with digestion problems and a sample group of 50 volunteers reflected the impartiality of the chosen TNS AISA agency and complied fully with our criteria.

The short time limit presented a challenge, as it did not allow us to carry out the study on a larger scale (more participants with digestive problems and more volunteers for stool sampling, wider diagnostics, etc.).

The respondents were at the age group from 18 to 55 years, in order to prevent possible distortion of information due to natural organic changes, improper digestion, or absorption in the gastrointestinal tract found in people over 55 years.

The study was focused on women because of the fact that the alimentary passage of women is demonstrably longer than that of men (*23*), and this fact influences more frequent incidence of indigestion. Women also exhibit better compliance not only with the filling in of the questionnaires, but in addition in their willingness to consume regularly and larger amounts of kefir beverage during longer periods than do men.

The method of stool sampling was selected to meet the requisite ethical criteria (which were recommended by the ethical committee). That is why sampling from the rectum was not promoted by the research team.

Storage and transportation of the samples corresponded with the standard norms and avoided any contamination.

The cultivation medium was selected with regard to the material equipment of the Department as well as the time scale. Due to the presence of the *Bifidobacterium animalis* strain in kefir beverages, it would certainly be more significant, from the point of view of the probiotic influence, to monitor the changes in the number of *Bifidobacterium animalis* in stool samples. Nonetheless, this was not possible for reasons specified above. Furthermore, there have already been a great many research studies focusing on the *Bifidobacterium animalis* strain, and these have established the favourable effects of that strain on human digestion in many cases (*9, 13, 14, 15, 24*). That is why we monitored the decrease in the bacterial *Enterococcus faecalis* strain in the stool samples of the group of volunteers. In the study carried out in vivo by *Huycke and Moore* (*25*), it had been shown that the *Enterococcus faecalis* strain produces hydroxyl radical. This indicates that this bacterium may be a potent source of oxidative stress on the intestinal epithelium. *Enterococcus faecalis* also produces extracellular superoxide. It can promote chromosomal instability in mammalian cells (*26*). Other studies also show that the decrease of the *Enterococcus faecalis* species is a visible sign of the favourable influence of the components of kefir beverages on the intestinal microflora of humans (*7, 27*). In our study, it has been proved that the counts of *Enterococcus faecalis* following a regular 14-day consumption of kefir beverage were reduced with statistical significance and thus have an effect on the intervened individuals' digestion.

We did not know the exact number of all bacteria in kefir beverage because it is non-public know-how of the Danone company. And it was not the aim of our study to find this out by ourselves, among other things for reasons of time and finance.

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Table 1

The results of the microbiological stool analysis before and after kefir beverage consumption

| Sample No.	SLANETZ BARTLEY *Enterococcus faecalis,* CFU/g^{-1} stool			
	Before	After	Decrease	Increase
1	$2.5.10^8$	$1.4.10^5$	1	0
2	$9.0.10^7$	$2.0.10^5$	1	0
3	$4.0.10^8$	$1.1.10^6$	1	0
4	$9.6.10^8$	$4.9.10^7$	1	0
5	$3.7.10^6$	$5.0.10^4$	1	0
6	$3.9.10^8$	$1.4.10^6$	1	0
7	$3.5.10^8$	$4.0.10^7$	1	0
8	$4.8.10^7$	$6.4.10^5$	1	0
9	$3.1.10^8$	$1.3.10^6$	1	0
10	$6.8.10^7$	$6.3.10^7$	1	0
11	$8.0.10^6$	$2.1.10^7$	0	1
12	$5.1.10^6$	$6.0.10^4$	1	0
13	$2.1.10^7$	$1.3.10^7$	1	0
14	$2.6.10^8$	$6.0.10^6$	1	0
15	$7.4.10^6$	$6.5.10^4$	1	0
16	$6.3.10^6$	$4.0.10^6$	1	0
17	$5.0.10^9$	$1.8.10^7$	1	0
18	$4.0.10^6$	$7.0.10^7$	0	1
19	$6.4.10^7$	$2.8.10^8$	0	1
20	$2.1.10^9$	$2.4.10^6$	1	0
21	$3.5.10^7$	$8.0.10^5$	1	0
22	$5.0.10^5$	$5.9.10^5$	0	1
23	$7.5.10^8$	$1.5.10^5$	1	0
24	$3.3.10^9$	$2.2.10^7$	1	0
25	$6.0.10^7$	$4.0.10^7$	1	0
26	$5.2.10^7$	$1.7.10^7$	1	0
27	$4.6.10^6$	$2.9.10^6$	1	0
28	$5.3.10^5$	$1.4.10^4$	1	0
29	$5.3.10^7$	$2.0.10^5$	1	0
30	$3.6.10^7$	$5.6.10^5$	1	0
31	$1.2.10^8$	$5.9.10^6$	1	0
32	$2.7.10^8$	$4.0.10^4$	1	0
33	$6.0.10^9$	$9.7.10^7$	1	0
34	$1.6.10^8$	$1.1.10^7$	1	0
35	$1.0.10^6$	$4.0.10^6$	0	1
36	$1.0.10^8$	$2.2.10^8$	0	1
37	$1.9.10^6$	$1.2.10^5$	1	0
38	$8.2.10^7$	$4.0.10^7$	1	0
39	$1.7.10^6$	$5.0.10^8$	0	1
40	$8.0.10^6$	$1.7.10^5$	1	0
41	$1.2.10^7$	$1.0.10^8$	0	1
42	$5.8.10^7$	$3.6.10^5$	1	0
43	$5.7.10^6$	$3.0.10^5$	1	0
44	$1.0.10^7$	$2.8.10^6$	1	0
45	$2.7.10^7$	$4.0.10^5$	1	0
46	$2.7.10^7$	$8.7.10^6$	1	0
47	$2.9.10^8$	$1.1.10^7$	1	0
48	$1.3.10^7$	$2.0.10^5$	1	0
49	$5.8.10^8$	$6.3.10^5$	1	0
50	$2.6.10^7$	$2.5.10^6$	1	0
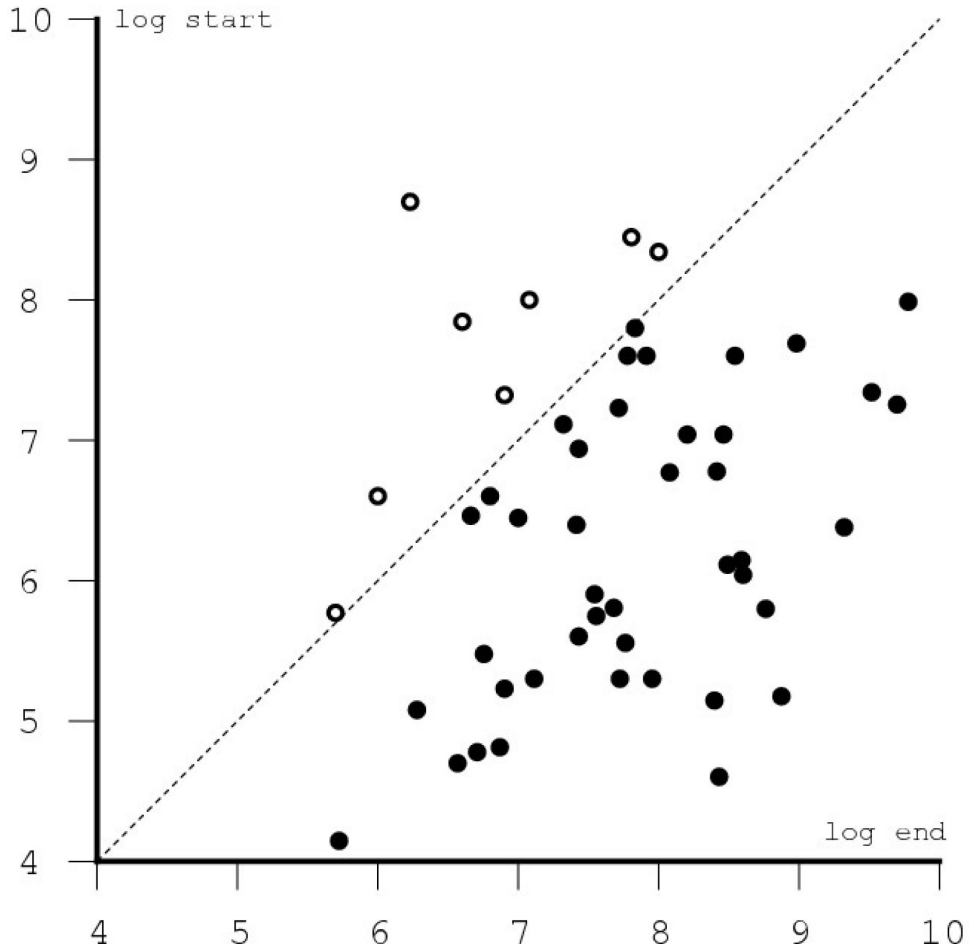
Fig. 1
Area distribution of individual respondents
Axis x: common logarithm of CFU concentration after intervention.
Axis y: common logarithm of CFU concentration before intervention.
Dashed line: points where concentration before and after intervention was the same.
Full circles: patients exhibiting higher CFU values before than after intervention.
Empty circles: patients exhibiting lower CFU values before than after intervention.