SYNBIO TECH Report

Intake of multistrain probiotic isolated from kefir improves gastrointestinal functions in human clinical trial

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"Kefir has traditionally been regarded as a beverage with added health benefits and consequently perhaps one of the oldest probiotic food. The well-knew villager with the secret of longevity drink kefir to improve the gut health."

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Better Probiotic Better Life

ABSTRACT

Kefir has traditionally been regarded as a beverage with added health benefits and consequently perhaps one of the oldest probiotic food. Seven strains of lactic acid bacteria were isolated from kefir and recombined to give a defined strain powdered kefir product "AB-kefir". The purpose of this study was to evaluate the effect of AB-kefir on gastrointestinal functions of healthy volunteers by questionnaire and checking the microflora of their feces. Fifty four participants, aged 18-40, were randomized into two groups receiving daily 4 grams of either placebo (n=27) or probiotic ($1x10^{10}$ CFU/g) (n=27), respectively. The microflora had no significant change during the experiment period comparing either with run-in period of probiotic group or with placebo group. The gastrointestinal functions were evaluated by questioning if participants' fecal consistency, diarrhea frequency, abdominal pain, appetite, choking and difficulty in swallowing, and feeling of nausea and vomiting were significantly improved after 3 weeks of ingestion of AB-kefir comparing to run-in period. It was demonstrated that the defined strain powdered kefir product "AB-kefir" could improve gastrointestinal functions and appetite on healthy individuals.

INTRODUCTION

Kefir, with a slightly sour, alcoholic and sparkling mouth feel, has been a traditional fermented milk produced for thousands of years in mountain villages of North Caucasus and steps from Northeastern Caucasus to Mongolia and spread from here to other countries including Taiwan. Traditional kefir is produced by the inoculation of kefir grains into milk from cows, goats, sheep, camels, or buffalos in goat skins bag, clay pots, or wooden buckets followed by a fermentation period of about 1 day at room temperature. Numerous species of bacteria and yeasts have been isolated from kefir grains and from the fermented kefir product (Table 1, Rattray FP, 2011). The microflora of kefir and kefir grain was predominated by lactobacilli and lactococci followed by yeast and acetic acid bacteria. Lactic acid bacteria are mainly responsible for the conversion of lactose to lactic acid, resulting in a pH drop and thus aiding preservation of the milk. Homofermentative lactic acid bacteria in kefir consist of lactobacilli such as *Lactobacillus delbrueckii* ssp. *bulgaricus*, *L. helveticus*, *L. kefiranofaciens*, *L. kefirgranum*, and *L. acidophilus*; lactococci such as *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*; and *Streptococcus thermophilus* (Table 1, Rattray FP, 2011). The heterofermentative lactic acid bacteria produce lactic acid and CO_2 from the fermentation of lactose, and include lactobacilli such as *L. kefir*, *L. parakefir*, *L. fermentum*, and *L. brevis*. The heterofermentative lactococci and the citrate-fermenting species such as the citrate-positive strains of *Lc. lactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, and *Leuconostoc mesenteroides* ssp. *mesenteroides* are of special interest (Rattray and O'Connel, 2011).

The reported scientific benefits of kefir include antipathogenic activity, antitumor and anticarcinogenic activity, alleviation of lactose maldigestion, enhanced synthesis of certain B vitamins, anti-inflammatory/immune modulation effects, and hypocholesterolemic effects. However, the inherent microbial variability of kefir grains and the different processes used in kefir manufacture result in inconsistent probiotic properties. Therefore, a consistent composition and cell count will be very important to commercialize a kefir product.

In our previous studies, seven strains of lactic acid bacteria were isolated from kefir and recombined to give a powdered kefir product with defined strains that give a consistent composition and cell count, and could be used as a dietary supplement. For evaluating it probiotic properties, we conduct a clinical trial of 54 volunteers in a medical center on gastrointestinal functionalities by questionnaire and analyze the microflora of their feces in this study.

"Traditional kefir is produced by the inoculation of kefir grains into milk from cows, goats, sheep, camels, or buffalos in goat skins bag, clay pots, or wooden buckets followed by a fermentation period of about 1 day at room temperature."

Table 1. Microflora identified in kefir grains (Rattray FP, 2011)

Lactobacilli	*Lactococci* and *Leuconostoc spp.*	*Yeasts*	Others
Homofermentative	***Homofermentative***	***Lactose-fermenting***	
Lb. acidophilus	*Lc. lactis spp. lactis*	*Kluyveromyces marxianus* (t)/ *Candida kefyr* (a)	*Streptococcus thermophilus*
Lb. delbrueckii spp. bulgaricus	*Lc. lactis spp. cremoris*	*Kluyveromyces lactis* var. *lactis*	*Acetobacter acrti*
Lb. helveticus		*Debaryomyces hansenii* (t)/ *Candia famata* (a)	*Acetobacter rasens*
Lb. kefiranofaciens		*Dekkera anomala* (t)/ *Brettanomyces anomalus* (a)	*Enterococcus durans*
Lb. kefirgranum			*Galactomyces geotrichum* (t)/ *Geotricum candium*(a)
Heterofermentative	***Heterofermentative***	***Non-lactose-fermenting***	
Lb. kefir	*Lc. lactis*(citrate-positive)	*Saccharomyces unisporus*	
Lb. parakefir	*Leu. mesenteroides ssp. cremoris*	*Saccharomyces turicensis*	
Lb. brevis	*Leu. mesenteroides ssp. mesenteroides*	*Saccharomyces cerevisiae*	
Lb. plantarum	*Leu. mesenteroides ssp. dextranicum*	*Saccharomyces exiguus*	
Lb. casei ssp. casei		*Saccharomyces pastorianus*	
Lb. paracasei ssp. paracasei		*Pichia fermentans* (t)(a)/*Candida firmetaria*	
Lb. fermentum		*Torulaspora delbrueckii*	
Lb. rhamnosus		*Candida friedrichii*	
Lb. fructivorans		*Candida humilis*	
Lb. hilgardi		*Issatchenkia orientalis*(t)/ *Candida krusei*(a)	
		Candida maris	
		Debaryomyces occidentalis	
		Yarrowia lipolytica (t)/*Candida lipolytica*	

(t):teleomorph (sexual reproductive stage); (a): anamorph (asexual reproductive stage).

MATERIALS AND METHODS

Materials

AB-kefir, containing seven strains of lactic acid bacteria (*Bifidobacterium longum*, *L. acidophilus*, *L. fermentum*, *L. helveticus*, *L. paracasei*, *L. rhamnosus*, and *Streptococcus thermophilus*) isolated from kefir, is a powder form lyophilized product with defined composition and cell count $1x10^{10}$ CFU/g. Placebo consisted of granulated corn starch whereas study preparation -AB-kefir powder was added in the dose of $1x10^{10}$ CFU/g of final product were dispensed in aluminum foil sachets.

Experimental Design

The study was conducted at the Department of Internal Medicine, Cheng Kung University Medical Center, Tainan, Taiwan, as a randomized, controlled single-blind, parallel 4 weeks trial, with a 1 weeks run-in period, 3 weeks of intervention and 1 weeks wash-out. Fifty four participants, aged 18-40, were randomized into two groups receiving daily 4 grams of either placebo (n=27) or AB-Kefir powder (n=27) after meal, respectively. The study design with the schedule is shown in Figure 1. At enrollment the participants received written and verbal information about the objectives of the study, the inclusion and exclusion criteria and the importance of avoiding fermented milk products and supplements with probiotics during the 5 weeks trial period. Otherwise, they were instructed to live and eat as they used to. Furthermore, they were explained how to fill in the questionnaire. At all five visits the participants were asked about changes in their wellbeing and whether they had been ill or had taken any kind of medication since the last visit. The protocol was approved by Institutional Review Board of National Cheng Kung University Hospital, protocol number BR-100-039.

Questionnaire survey

Subjects were asked to enter in questionnaire sheet regarding to fecal consistency, appetite change and wellness after intervention every visit.

Fecal samples

At visits 2 to 6 the participants delivered one fresh (<24 h) fecal sample in a plastic container in a zipper bag. At home the container was stored in a cool-bag containing

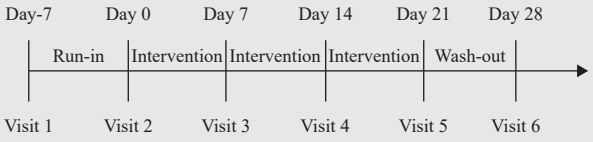


Fig 1. Design of the study with run-in, invention and washout phase along with visits and sample collection

two frozen chill elements and transported to the department, where they were stored at 5°C for up to 3 h before further analysis.

Microbiological Analysis

Microbiological analysis of the sample feces was according to the method issued by Taiwan Food and Drug Administration. Fecal samples were tested in appropriate dilutions on specific agars for the number of clostridia (TSC Oxoid CM587+SR88, anaerobic incubation 20 to 24 h at 35°C), *bifidobacteria* (MPN medium (Tanaka and Mutai,1980) anaerobic incubation 48 to 72 h at 35°C) lactobacilli (de Man, Rogosa and Sharpe agar Difco; anaerobic incubation 48 to 72 h at 35°C) anaerobic bacteria (CDC agar, Becton Dickinson, Cockeysville, MD, anaerobic incubation 48 to 72 h at 35°C), aerobic bacteria (BAP agar, Becton Dickinson, Cockeysville, MD, aerobic incubation 16 to 18 h with 5% CO_2 at 35°C), *Staphylococcus aureus* (Mannitol salt agar, aerobic incubation 16 to 18 h at 35°C) and *E. coli* (Eosin-Methylene Blue agar, Sigma-Aldrich, USA, aerobic incubation 16 to 18 h at 35°C).

Statistical Analysis

Results are presented as mean ±SD. The statistical significance of differences was examined by Student's t-test and $p<0.05$ were regarded as significant. Chi-square test or Fisher's exact probability test was used to analyze frequency of occurrence.

RESULTS AND DISCUSSION

Bacterial Profile

No significant changes in the fecal microflora between the AB-kefir group and the placebo group could be detected during the experiment period (Fig 2). As it was not practically possible to receive the fecal samples immediately after 'delivery', we had to decide how to store the samples. Freezing them immediately after 'delivery' would probably change the fecal composition and kill some bacteria and cold storage up to 24 h would probably also changes the bacterial composition to some degree; however, the method used in this study was expected to be the best and therefore chosen. Choosing the media to analyze the specific bacterial groups upon, created another double-edged decision since selective media are known to inhibit the wanted bacteria to some degree too. In this way, various selective media underestimates some bifidobacteria species and fails to recover others (Apajalahti *et al.*, 2003). The high initial number of *Bifidobacterium* (around 8.0-8.3 $\log_{10}$ CFU) in the volunteers may be another reason why the effect of AB-kefir on the *Bifidobacterium* number was not marked. The small microbial changes observed in the study may reflect a stable microflora in

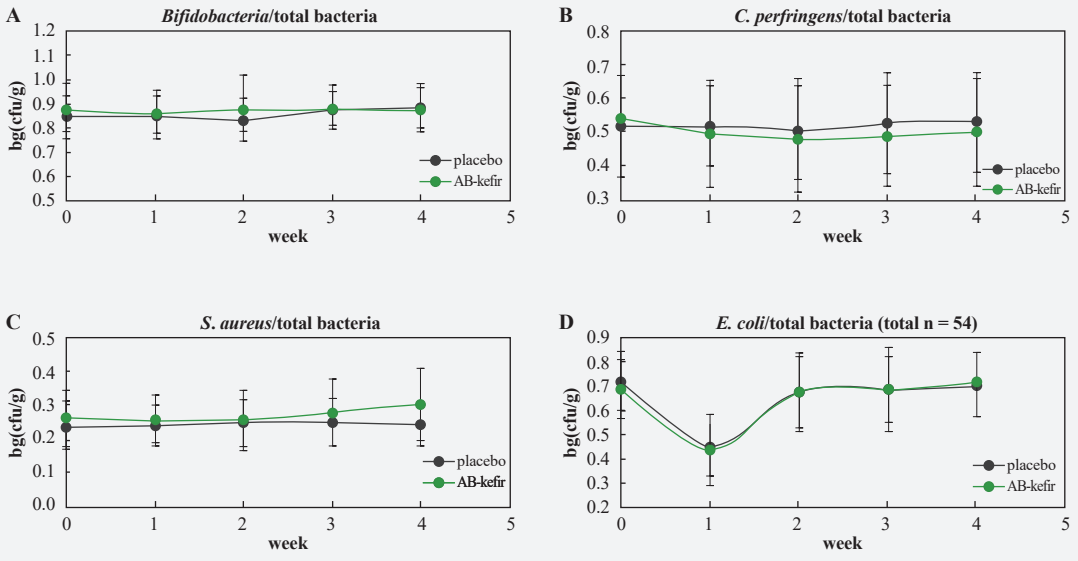
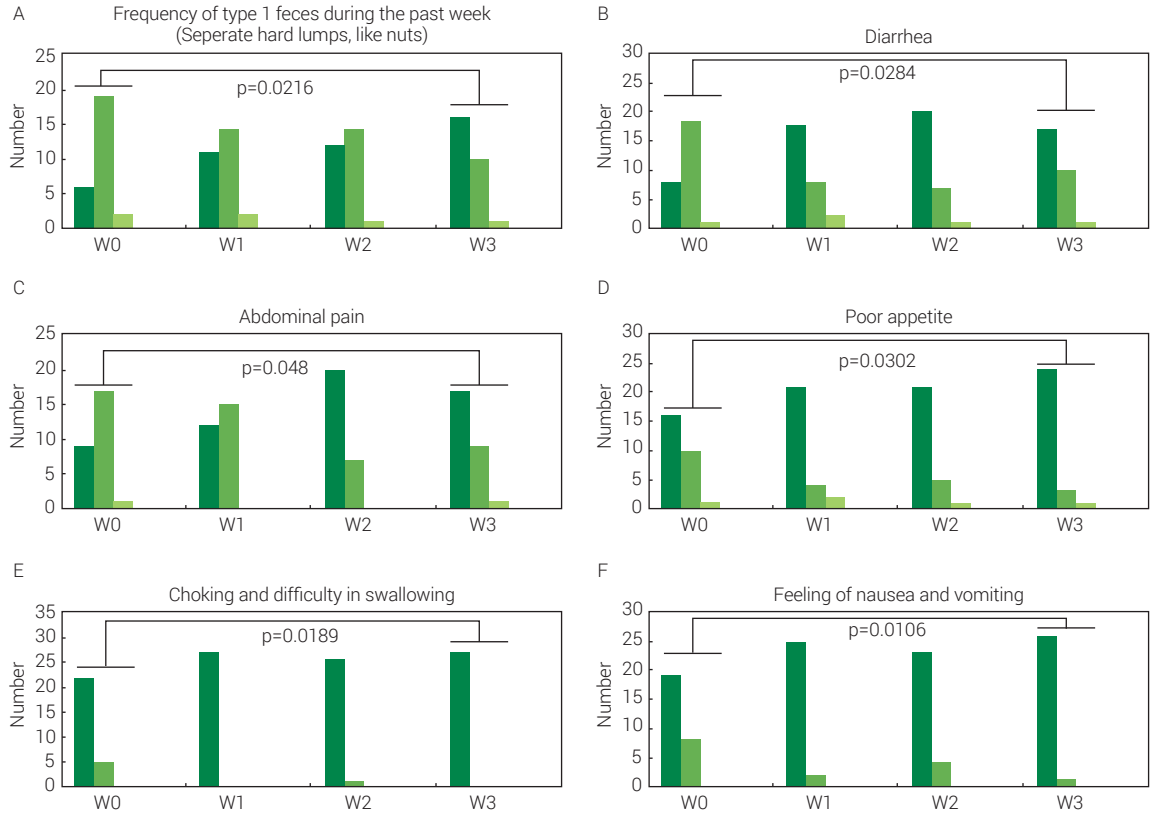


Fig 2. Changes in the bacterial profile before and after the intervention for the placebo group and the AB-kefir group. Total bacteria were the sum of aerobic and anaerobic bacteria count.

our healthy subjects (Ogata *et al.*, 1999).

Gastrointestinal Functions

Gastrointestinal functions before and after intervention of probiotic were evaluated by questionnaire weekly. Fecal consistency was recorded according to the illustrations from the Bristol scale (Heaton and Thompson, 1999). Frequency of type 1 feces (nuts like) was reduced after ingestion of AB-kefir, and was significantly reduced after 3 weeks of ingestion (p=0.0216) (Fig 3A). The number of volunteers who had the symptoms of diarrhea, abdominal pain, choking and difficulty in swallowing, feel of nausea and vomiting, and poor appetite were reduced after ingestion of AB-kefir, and was significantly reduced at third week (W3) compared to run-in period (W0) (p=0.0284, 0.048, 0.0189, 0.0106, and 0.0302, respectively) (Fig 3B, 3C, 3E, 3F, and 3D). Some studies indicate that probiotics can influence the bowel habits of healthy individuals (Alm *et al.*, 1993; Marteau *et al.*, 2002; Xiao *et al.*, 2003) as well as of people suffering from diarrhea. In the present study, healthy individuals before ingestion of probiotic reported to have more frequency of solid stools compared to the intervention period. The present study also revealed a tendency of reduction in the frequency of diarrhea with the ingestion of probiotics. These effects on the bowel function have been reported elsewhere too, for example in a study with healthy individuals receiving a milk product fermented with *B. longum*, where one of 16 persons in the placebo group and 5 of 16 persons in the supplemented group experienced increased fecal frequency in the intervention period (Xiao *et al.*, 2003). Another study showed that elderly constipated individuals experienced a significant improvement of bowel movements after consumption of fermented milk (P<0.05) supplemented with BB-12 and *L. acidophilus* LA-5 (Alm *et al.*, 1993). One of the most interesting findings in this study was the ingestion of AB-kefir could alleviate troublesome symptoms like abdominal pain, choking, difficulty in swallowing, and feeling of nausea and vomiting, that maybe not a disease, but really reduce the quality of life. The other interesting finding was ingestion of AB-kefir could improve appetite. Liu and his colleagues studied the effects of probiotic yogurt on intestinal flora of patients with chronic liver disease and found the intervention of probiotic yogurt significant improved food intake, appetite, abdominal distension, and ascetic fluid (Liu *et al.*, 2010). Many studies



Comparison of gastrointestinal functions before and after of AB-kefir intervention. Symptoms were scaled by frequency occured every week as: Never, 1 day per week, 2-3 days per week, 4-5 days per week, Every day, and the number of subjects with the same frequency were sum up (N=27).

"AB-Kefir strains were isolated from kefir grains as an ingredient of probiotic preformulated blend. It is approved to improve gut health through human clinical trial and has been applied to various health care products."

also demonstrated that probiotic treatment mitigate abdominal pain in patient with irritable bowel syndrome (Drouault-Holowacz *et al.*, 2008; Ducrotte *et al.*, 2012;Guandalini e*t al.*, 2010; Hun L, 2009;Kajander *et al.*,2008), but this is the first time of the present study to show defined strain powdered kefir product improves gastrointestinal functions and appetite on healthy individuals.

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