Official Title of the study: Effect of Kefir on Appetite

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1. Background

Obesity is a global public health problem. According to World Health Organization (WHO) 2016 data, approximately 600 million people are obese and 2 billion people being overweight (1). A low glycemic index (LGI) diet recommended as an alternative dietary approach in the treatment of obesity and impaired glucose homeostasis (2). When LGI foods are consumed instead of high glycemic index (HGI) foods, glycemic control can be improved (3). In addition, the type of meal plays an important role in energy intake and body weight management (4). Diet also plays an important role on microbiota in the development of obesity. For this reason, gut microbiota modulation using pre- and probiotics is drawn attention (5). Probiotics can help lose weight by providing appetite control (6), and reducing energy intake (7). In recent years, studies conducted on obesity therapy have shown that gut microbiota plays a role on diet-induced obesity in animals and also modifies insulin resistance, inflammation and immunological response (8, 9). It has been also found that microbiota has direct or indirect effects on energy balance and appetite control in rats (10, 11). Furhermore microbiota can play an important role in gastrointestinal system function and in harvesting diet energy (12).

The influence of gut microbiota on the glucose homeostasis has recently begun to attract attention (13). However effects of probiotics on glucose homeostasis are not clear yet (14). One of the possible mechanisms is thought to be the prevention or delay of the inflammatory process by changing the bacterial diversity of the intestinal bacteria via use of probiotics (15). Furthermore, the effects of the probiotics on the antioxidant system and the hormones secreted from the gut lumen are also influential on glucose homeostasis (16). Another mechanism may be that probiotics affect insulin sensitivity (17). In previous studies, it was observed that probiotic supplementation significantly reduced insulin levels (16, 18), fasting glucose and postprandial glucose (19), and improved insulin sensivity (18, 20).

2. Objective

The aim of this study was to determine whether the kefir, a natural probiotic, could affect postprandial glycemic responses and appetite sensations in healthy subjects, consuming three test meals that had similar energy content but different GI amounts and milk or kefir.

3. Materials and methods

3.1. Participants

Twenty four healthy, normal-weight (BMI 18.5–25 kg/m^2) females, aged 21–24 years, were recruited from Erciyes University and the surrounding community. Exclusion criteria were following an energy-restricted diet during the last three months, change in body weight >5 kg during the last three months, lack of appetite, physician-diagnosed conditions or medications that influence metabolism, having any chronic diseases such as diabetes, hypertension etc., fasting glucose greater than 100mg/dl, smoking, practicing endurance sports, having difficulties with swallowing/eating, hypersensitivity for the food products under study, being a vegan, and pregnant or lactating. Furthermore, one enrolled participant declined to eat other test breakfasts after consuming first test breakfast, and the other one had influenza during study period was

excluded because of a possible effect of infection on postprandial glucose response and appetite. Finally, leaving a total of 22 participants that were assessed for the main outcomes (Figure 1).

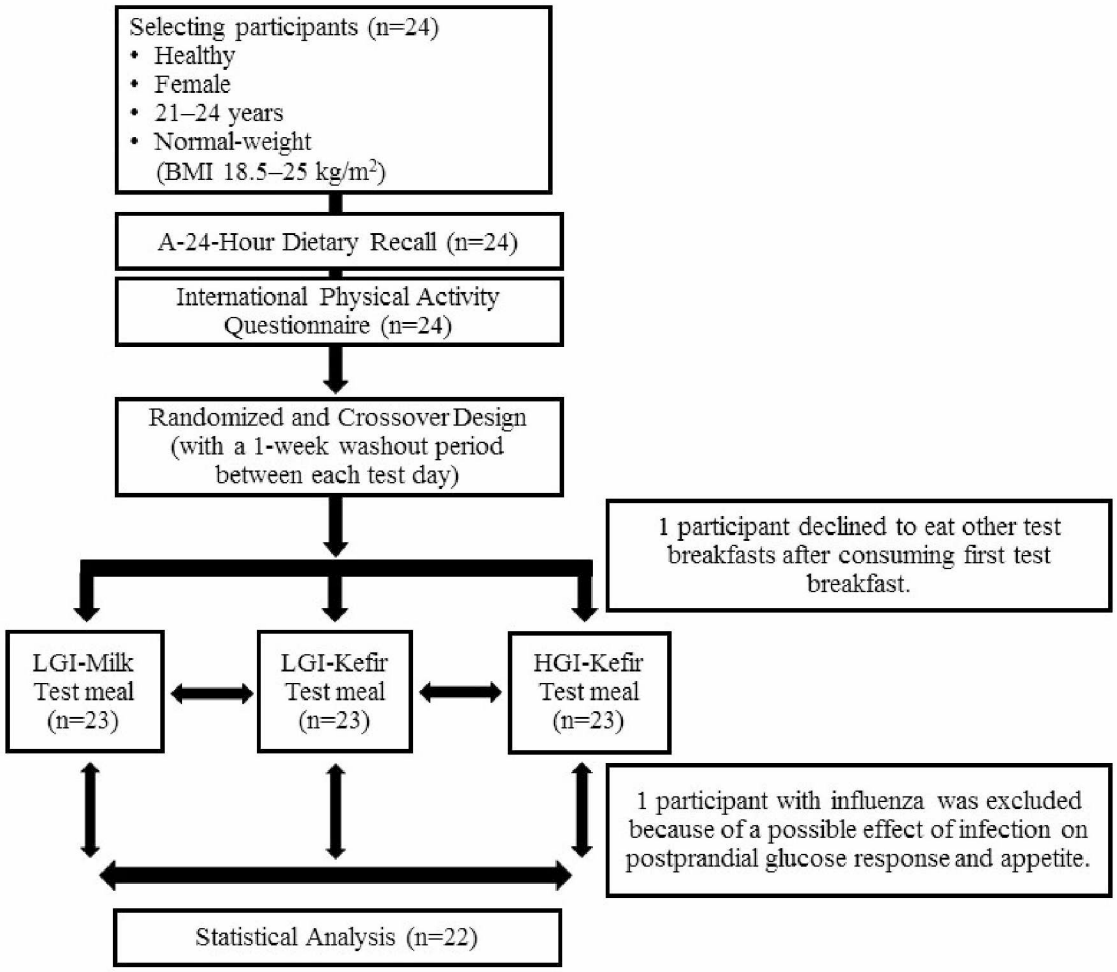

Figure 1. Participant recruitment flow diagram

3.2. Nutritional evaluation

Body height and weight of participants were obtained and BMI was calculated. Waist and hip circumference were measured using a non-elastic tape with the participants standing, with the face directed towards, shoulders relaxed, and the tape was positioned at a level parallel to the floor.

Participants' dietary intakes were assessed by the a-24-hour dietary recall using a photographic atlas of food portion sizes to quantify the data in the beginning of study and on the day of each test meal (21). Diet composition was analyzed by the BeBiS Nutrition Information System software version 7.2 (22).

Physical activity level was evaluated simultaneously with dietary assessment by the International Physical Activity Questionnaire (IPAQ) short form, a validated survey instrument (23). The 7-item IPAQ records self-reported physical activity in the last seven days. Responses

were converted to Metabolic Equivalent Task (MET) minutes per week according to the IPAQ scoring protocol (24).

3.3. Study design

This was a randomized, single-blind, 3-intervention crossover trial conducted on 3 separate days, with a 1-week washout period between each study day (21). All participants were randomly submitted to three different test meals with the following different GI amounts and milk or kefir: a low GI and milk content (LGI-Milk), a low GI and kefir content (LGI-Kefir), and a high GI and kefir content (HGI-Kefir). The order of the test meals was determined by using a computer-generated randomization sequence before recruitment. The primary outcomes were postprandial response of plasma glucose and appetite sensations. Desire for specific food types, palatability of test meals and subsequent food intake were the secondary outcomes.

Participants received each test meal in a randomly assigned order on three different mornings separated by a washout period of 1-week when they were asked to maintain their usual diet and physical activity (25). Breakfast was preferred as a test meal because postprandial responses were more prominent in the morning. On the day before each test meal, participants were instructed to eat a standard evening meal at 21:00 h and to refrain from eating and/or drinking (except for water) and/or doing any physical activity beyond that of their typical daily activities (26). Moreover, participants were tested within the follicular phase of their menstrual cycle (3–10 d after onset of menses) to avoid possible influences of menstrual cycle phase on hormonal changes and appetite (27).

On the each testing day, participants arrived in the testing room at 08.30 h following a 12-h fast and anthropometric measurements were completed before eating the test meal. Also, first blood glucose level and appetite scores were measured for baseline measurements (time zero). At 09:00 h participants received the test meal blinded to its nutritional characteristics and were asked to consume within 15 min (27). During the postprandial period, participants remained at rest in the testing room and their capillary blood glucose concentrations were obtained with the use of the finger stick method (glucometer; Accu-Check, Roche Diagnostics) at time points 30, 60, 90, 120, and 180 min (21). Moreover, appetite scale was applied at 15, 30, 60, 90, 120, 150 and 180 min and participants were asked to assess the palatability (visual appeal, smell, taste, aftertaste and overall palatability) of test meal at 15 min (immediately after consuming test meal) (28) (Figure 2). No food or drink other than water was allowed following consumption of the test meal until an *ad libitum* lunch. Water was available *ad libitum* throughout the first trial; however, the volume consumed was measured and the participants drank the same volume during the second and third trial (29). Participants were permitted to watch movies, read, or play with electronic devices (laptop computer, mobile phone etc.) or undertake other similar sedentary activities throughout each test day but were not allowed to sleep (26).

This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures were approved by the Clinical Research Ethics Committee of the Erciyes University (2016/547) on 21 October 2016, and all participants gave written informed consent.

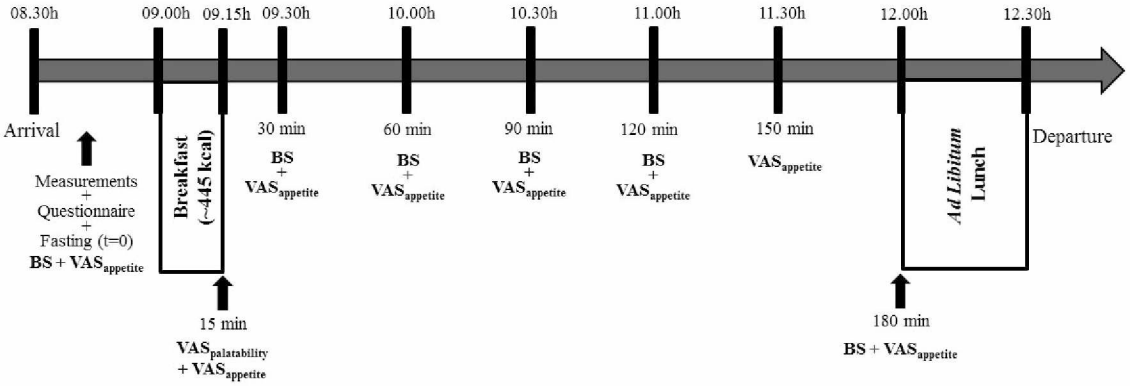

Figure 2. Study protocol and timeline on test days

3.4. Test meals composition

All test meals were matched for energy, LGI-Milk and LGI-Kefir meals also for macronutrients and GI, but HGI-Kefir meal had a nearly 2-fold difference in GI (Table 1). Furthermore, both milk and kefir drinks contained 120mg/100ml calcium, and kefir drink also had 10^7 CFU/g probiotic bacteria (Lactobacillus spp. and Streptococcus spp.). The energy content of test meals (~445 kcal) were estimated as corresponding to about 25% daily energy needs of a sedentary female (27), and the daily energy needs were also calculated by the Schofield equation (, taking into account: gender, age, weight and a physical activity level of 1.3.

Table 1. Nutritional composition and GI of the component foods in test meals

	Portion size (g/ml)	Energy (kJ[kcal])	AvCHO* (g)	Protein (g)	Fat (g)	GI (%)
LGI-Milk						
Milk (full-fat)	200	524(125)	9.9	5.8	6.8	31
Cheddar cheese	25	424(101)	0.0	6.4	8.5	0
Apple	200	469(112)	24.9	0.9	0.9	36
Grain bread	50	435(105)	19.1	4.8	0.9	50
Toplam		**1852(443)**	**53.9**	**17.9**	**17.1**	**40**
LGI- Kefir						
Kefir (full-fat, plain)	200	526(126)	10.0	5.8	6.3	36
Cheddar cheese	25	424(101)	0.0	6.4	8.5	0
Apple	200	469(112)	24.9	0.9	0.9	36
Grain bread	50	435(105)	19.1	4.8	0.9	50
Toplam		**1854(444)**	**54.0**	**17.9**	**16.6**	**41**
HGI- Kefir						
Kefir (full-fat, plain)	200	526(126)	10.0	5.8	6.3	36
Raspberry jam	50	568(136)	31.8	1.7	0.0	78
White bread	75	773(185)	34.0	7.0	1.5	70
Toplam		**1868(447)**	**75.8**	**14.5**	**7.9**	**70**

*AvCHO, available carbohydrate including sugars and starch, excluding fiber.

GI of foods in test meals was estimated by using the GI tables, with glucose as the reference food (30). The average meal GI was calculated as follows (31).

$$\text{Meal GI} = \frac{\sum_{a=1}^{n} (GI_a \times AvCHO_a \times Frequency_a)}{\sum_{a=1}^{n} (AvCHO_a \times Frequency_a)}.$$

where n is the number of foods consumed, GI_a is the GI for food a, $AvCHO_a$ is the available carbohydrate content per serving of food a, and $Frequency_a$ is the consumption frequency of one serving of food a during the meal.

3.5. At libitum lunch

At 180 min after the test breakfast, participants were presented with the *ad libitum* lunch following blood glucose and appetite sensation measurements. Frequently consumed foods were used to prepare the lunch consisting of pasta with tomato sauce, yogurt drink and mandarin. Participants were asked to consume whatever they wanted and to eat until they felt comfortably full (26). Foods were weighted or measured to the nearest 0.1 g before consumption, and any remaining food was reweighed to determine intake at lunch. Energy and macronutrient values were calculated using The National Food Composition Database (TurKomp) (32), and manufacturer labelling. All foods served at breakfast and lunch were prepared by the research dietitian in the kitchen of the Nutrition Laboratory of the University on the day of each test meal.

3.6. Assessment of appetite sensations

Subjective assessment of appetite sensations was performed by using a visual analog scale (VAS) composed of lines (of 100 mm in length) with words anchored at each end, expressing the most positive and the most negative rating. VAS was used to assess appetite scores (hunger, fullness, desire to eat, and prospective food consumption), desire for specific food types (sweet, salty, savoury, and fatty) and the palatability of test meals (visual appeal, smell, taste, after taste, and overall palatability) (28). As shown in Figure 2, a series of VAS were administered using the paper-and-pen method at specific time-points during the examination period. Participants were asked to make a single vertical mark at the appropriate point between the 2 anchors on each scale corresponding to their feelings. A new VAS booklet was provided to participants for each rating time, and nobody could compare to his/her previous ratings when marking the VAS. Appetite scores were quantified by measuring the distance in millimeters between the left end of each line and the mark (21). Furthermore, the separate VAS components such as hunger, fullness, desire to eat and prospective food consumption were combined to produce an additional measure termed 'composite appetite score'. This validated average appetite score was calculated for each time point using the following equation: [(hunger + desire to eat + prospective food consumption + (100 - fullness)]/4 (33).

4. Statistical Analysis Plan

4.1. *Sample size*

A power-based sample size calculation based on previous research from Stevenson *et al.* (29) revealed that 21 participants were needed to provide 80% power to detect 5% difference between groups in primary outcomes. To allow discontinuation during the study, 24 participants (considering 15% losses) were recruited, and the study was concluded with 22 participants.

4.2. Data analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (version 22.0; IBM SPSS Statistics) software. Data were expressed as the mean±SD or mean (95% CI) unless otherwise indicated. Normality was assessed using the histogram and normal Q-Q plots, and also Shapiro-Wilk test. Furthermore, continuous variables were examined for skewness and kurtosis, and log-transformed before analysis and reported back-transformed geometric means (G) ± standard error (S.E) when required (34). Postprandial glucose responses and appetite sensations were quantified as area under the curve (AUC) calculated according to the trapezoidal rule (21). One-way (1-factor) analysis of variance (ANOVA) for repeated measures was applied to determine statistical differences between groups. In addition, the data were analyzed by using 2-factor (time x meal) repeated-measures analysis of variance (ANOVA), and Bonferroni post hoc tests were applied to significant time x meal interactions. For all statistical analyses, p values less than 0.05 were considered to have statistical significance (35).

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