DETERMINATION OF THE PRESENCE OF LIVING MICROORGANISMS IN KEFIR GRAIN

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ABSTRACT
Kefir, defined as fermented milk product, is a drink that lactic acid bacteria and yeasts formed together with kefir grain obtained by adding milk and waiting at room temperature for one day. Kefir is being investigated with increasing interest in recent years as it contains micro-organisms useful for health. Although kefir is produced on an industrial scale, consumers have traditionally been producing kefir in homes. This study aimed to determine the presence of living lactic acid bacteria and yeast in kefir grain. Man Rogosa and Sharp (MRS) media were used for isolation and counting of lactic acid bacteria and Yeast extract Glucose Chloramphenicol agar (YGC) was used for yeasts. Kefir grain was found to contain $5x10^4$ CFU/g of living lactic acid bacteria and yeast.

Keywords: *Probiotic, Lactobacillus, Yeast, Health benefits, Saccharomyces*

INTRODUCTION

Kefir is a milk-based fermented beverage produced using a micro-organism community called kefir grains or kefir cultures. Based on Turkish Food Codex **[2],** kefir is defined as a fermented milk product in which starter cultures or kefir grains are used in fermentation containing different strains of *Lactobacillus, Leuconostoc, Lactococcus* and *Acetobacter* species and strains of yeast species that are able to ferment (*Kluyveromyces marxianus*) or non-ferment(*Saccharomyces unisporus, S. cerevisiae, S. exiguus*) lactose. Nowadays, with the increasing importance of consumers to eat natural and healthy foods, the demand for kefir also increases. This increase in consumer demand has accelerated the commercialization of kefir, which was previously produced only in individually at homes. The source of kefir is Caucasus and Tibet, it started to be produced in Eastern and Central European countries towards the end of the century in 19th century and today it has spread all over the world.

The production of fermented foods has been done since ancient times (A.C. 7000-A.C. 8000) is known. However, commercially the production of meat and milk-based fermented foods increased after 1970. Kefir is one of the commercially produced fermented beverages in our country.

Fermented foods are produced using a wide variety of raw materials. These include a large number of fermented foods produced using vegetables and fruits, milk, meat, fish, and cereals. Examples of some fermented foods produced in our country are given in Table 1. Some foods, such as cheese, pickles, and wine, are more or less different in their sensory and structural properties but have been accepted all over the world. However, fermented fish is more preferred in northern Europe and America and Asian countries, while fermented rice is produced in Asia, fermented cereals (boza) in our country, the Caucasus, and the Balkans. Fermented foods such as pickles, vinegar, olives, sausage, and turnip, especially yoghurt, are traditionally produced individually at homes for years.

Table 1: Examples of fermented food and beverages and their raw material

Fermented Foods	Raw Materials
Boza	Wheat, barley, corn, millet, rice
Hardaliye	Black grapes, cherries, mustard (grain)
Kefir	Milk
Cheese	Milk (cow, sheep, goat)
Feta cheese Cheddar cheese Sheep cheese (Pergamon Erzincan, Izmir) Blue mould cheese Pot cheese Herbed cheese Hellim cheese	
Sausage	Meat
Vinegar	Greengrocery
Turnip	Turnips, black carrot
Pickle	Fruits and vegetables
Yoghurt	Milk
Olive	Black and green olives

Fermented foods in our country as a variety of the highest number of foods produced are cheese and pickles. The most common types of cheese are feta cheese, tulum cheese, and cheddar cheese, while cucumber, cabbage, and pepper pickles are the examples of pickles produced in the greatest amount of time. It is observed that vinegar, which is added as flavoring to food and sauces, has been consumed as a beverage in recent years considering it to be beneficial to health.

Literature related fermented foods have been found in numerous studies examined kefir. While some studies have examined the effect of the milk variety on the physical, chemical and sensory properties of kefir, the majority of research has focused on microbiota and kefir micro-organisms.

In addition to the milk of animals such as cows, sheep, and goats, there are also studies where camel and horse milk are used in the production of kefir. Vegetable-derived milk substitutes such as soy, rice, and coconut milk can also be used. The Kefir grain consists of a structure in which a group of micro-organisms, lactic acid bacteria and yeasts, cling to each other with their own polymeric substances. When a kefir grain is added into a liquid medium such as milk, the micro-organisms that make up the kefir grain act out of the cell by releasing various flavorings, especially organic acids such as lactic acid, acetic acid and CO_2. Thus, kefir is formed with a slightly acidic, distinctive taste, slightly frothy and creamy-white color. An article published by Altay et al. **[3]** about non-alcoholic fermented Turkish beverages has included a broad literature review on micro-organisms found in kefir. In the studies carried out in Table 2, the species belonging to the *Lactobacillus* genus and yeast species in Table 3, which were found in kefir samples.

Table 2: *Lactobacillus* species found in kefir

***Lactobacillus* spp. in Kefir**	**References**
Lactobacillus acidophilus	**[4, 5]**
L. brevis	**[6]**
L. casei	**[4]**
L. casei subsp. *pseudoplantarum*	**[7]**
L. delbrueckii subsp. *bulgaricus*	**[7]**
L. fermentum	**[5]**
L. helveticus	**[5]**
L. kefiranofaciens	**[6]**
L. kefiri	**[6]**
L. otakiensis	**[5]**
L. plantarum	**[8, 9]**
L. sunkii	**[5]**

Kefir contains different species of lactic acid bacteria belonging to the genus *Lactobacillus, Lactococcus, Pediococcus, Streptococcus* and *Leuconostoc*. Lactic acid bacteria is the name given to a group of bacteria that produce lactic acid and are proven not to be harmful to health. They are

considered GRAS (Generally Recognized as Safe) status because they are not harmful to health. The two new species as *Lactobacillus kefiranofaciens* and *L. kefiri*, which are not found in other fermented foods, have been found **[6].**

Table 3: Yeasts species found in kefir

Yeast species in Kefir	References
Candida inconspicua	**[7]**
C. kefyr	**[10]**
C. krusei	**[10]**
C. lambica	**[10]**
C. maris	**[7]**
Cryptococcus humicolus	**[10]**
Geotrichum candidum	**[10]**
Kazachstania aerobia	**[11]**
Kluyveromyces marxianus	**[9]**
Lachancea meyersii	**[11]**
Pichia fermentans	**[12]**
Saccharomyces cerevisiae	**[11]**
S. turicensis	**[12]**

The kefir composition is given in Table 4.

Table 4: Kefir composition and micro-organisms load **[1]**

Property	Quantity
Milk protein (w/w %)	min. 2,7
Milk fat (w/w %)	max. 10
Titration acidity (as lactic acid %)	min. 0,6
Ethanol (v/w %)	-
Total specific micro-organisms (CFU/g)	min. 10^7
Total additional micro-organisms indicated on the label (CFU/g)	min. 10^6
Yeasts (CFU/g)	min. 10^4

In a study by Renner and Renz **[13]**, amino acids, vitamins, and trace metals found in kefir were determined as well as the composition of kefir and given in Table 5.

Table 5: Chemical composition of kefir **[13].**

Components			
Fat (%)	3.5	**Trace metals (mg)**	
Protein (%)	3.3	Iron	0.05
Lactose (%)	4.0	Copper	12
Water (%)	87.5	Molybdenum	5.5
Essential amino acids		Zinc	0.36
Tryptophan	0.05	Manganese	5
Phenylalanine + Tyrosine	0.35	**Vitamins**	
Leucine	0.34	A (mg)	0.06
Isoleucine	0.21	Carotin (mg)	0.02
Threonine	0.17	B1 (mg)	0.04
Methionine + Cystine	0.12	B2 (mg)	0.17
Lysine	0.27	B6 (mg)	0.05
Valine	0.22	B12 (mg)	0.5
Mineral Matter		Niacin (mg)	0.09
Calcium (g)	0.12	C (mg)	1
Phosphorus (g)	0.1	D (mg)	0.08
Magnesium (mg)	12	E (mg)	0.11
Potassium (g)	0.15	Lactic acid (%)	0.8
Sodium (g)	0.05	Phosphatides (mg)	40
Chlorine (g)	0.1	Cholesterin (mg)	13
		Ethyl alcohol (mg)	0.2

It has been determined that kefir is rich in phenylalanine, leucine, lysine, valine and isoleucine amino acids and in vitamins B2, B12, E and its energy value is 65 kcal at 100 mL **[13]**.

Kefir is also rich in vitamin C. There are numerous articles about the positive effects of kefir on health. But some of these positive effects have not yet been proven. The beneficial effects of kefir on health are summarized below. The most important benefit of kefir besides its rich mineral and nutrient content is that it can be consumed by individuals with lactose intolerance. Kefir contains all the nutrients found in milk. Due to its unique composition and structure, milk is also a suitable environment for micro-organisms. Micro-organisms provide nutrients more easily in aqueous environments and multiply faster. A major part of lactose found in milk is biodegrading by bacteria. As a result, the amount of lactose decreases on the one hand, while the amount of lactic acid increases on the other. Similarly, some of the proteins are also biodegrading by bacteria. Thus, kefir becomes a product with properties that can be consumed by individuals who are lactose intolerant and allergic to milk proteins. Furthermore, biochemical changes that occur during fermentation enable kefir to have its own unique flavor properties.

The probiotic properties of some of the kefir bacteria also contribute positively to the intestine microbiota. Most of the health benefits attributed to kefir are associated with kefir containing high amounts of probiotic micro-organisms. Studies on the control and improvement of many diseases with the consumption of kefir are still ongoing and it is stated that it creates protective and therapeutic effects against these ailments by providing the balance of the stomach and intestinal flora with antimicrobial and anticancer effects **[14, 15, 16]**.

Kefir is receiving increasing interest from consumers due to its beneficial health effects. Although kefir is commercially produced, traditionally kefir production is also very common in homes. In this study, the presence of living bacteria in the kefir grain used to produce kefir at home was examined and the total number of bacteria, lactic acid bacteria, and yeast was determined for this purpose.

MATERIAL AND METHOD

Material

As a material, kefir grain that used in making kefir individually at home was used. Kefir grain of approximately walnut-size was added to 500 ml UHT milk and incubated at 25°C.

At the beginning of incubation and 3 and 6 hours after incubation, samples were taken and microbiological inoculation was done.

Determination of Total Number of Bacteria

Plate Count Agar (PCA, Oxoid) was used to determine the total number of bacteria. After the medium sterilized in an autoclave at 121°C and 15 min. it is cooled to 50°C and poured into sterile petri plates. Inoculation was done by the spread plate technique. The total number of bacteria (CFU/mL) was determined by incubating at 30°C.

Determination of The Number of Lactic Acid Bacteria

Man Rogosa and Sharp (MRS) agar were used to identify lactic acid bacteria. It was prepared as PCA and inoculation were done by pour plate technique.

Determination of The Number of Yeast

YGC medium is used to determine the number of yeasts. This medium was prepared as PCA. Inoculation was done by the spread plate technique.

RESULTS

The total number of bacteria, the number of lactic acid bacteria and the number of yeast were given in Table 7, in the kefir grain that was inoculated in milk initially, 3 and 6 hours after incubation.

Table 6: Total number of bacteria, lactic acid bacteria and yeast in kefir grain

Media	Incubation time (h)		
	0	3	6
PCA (CFU/g)	$2x10^5$	$6x10^5$	$6x10^6$
MRS (CFU/g)	$5x10^4$	$3x10^4$	$2x10^5$
YGC (CFU/g)	$5x10^4$	$3x10^4$	$5x10^5$

As can be seen, by the examination of Table 7, the total number of bacteria per gram was 10^5 CFU, while 6 hours later it increased to $6x10^6$ CFU. The change in the total number of bacteria due to time is given in Figure 1.

The number of lactic acid bacteria in kefir (Table 6) was initially $5x10^4$ CFU/g, but after 6 hours it increased to $2x10^5$ CFU/g, while the number of yeast increased from $5x10^4$ CFU/g to $5x10^5$ CFU/g.

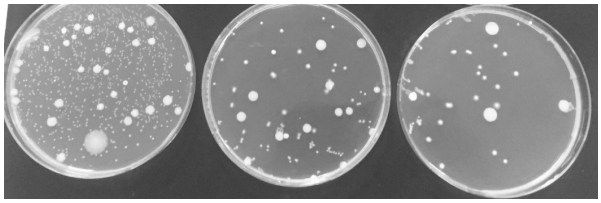

Figure 1: Colonies after 0, 3. and 6. hours incubation in PCA

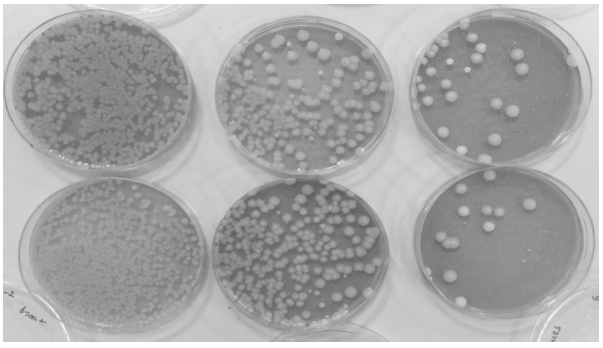

Figure 2: Lactic acid bacteria and yeast colonies at the end of 6 hours in MRS agar media

Figure 3 shows yeast colonies in YGC Agar media and Figure 4 shows yeast cells in light microscopy (40x).

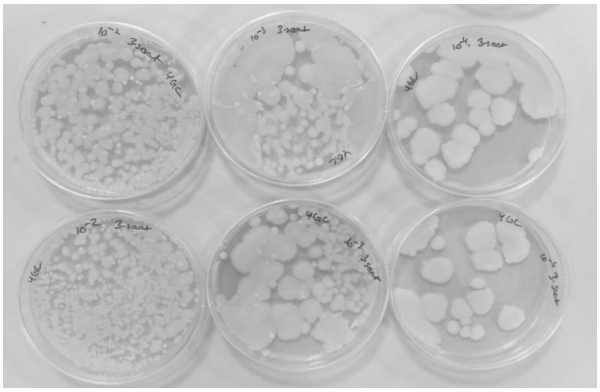

Figure 3: Yeast colonies in YGC (Parallel Inoculation)

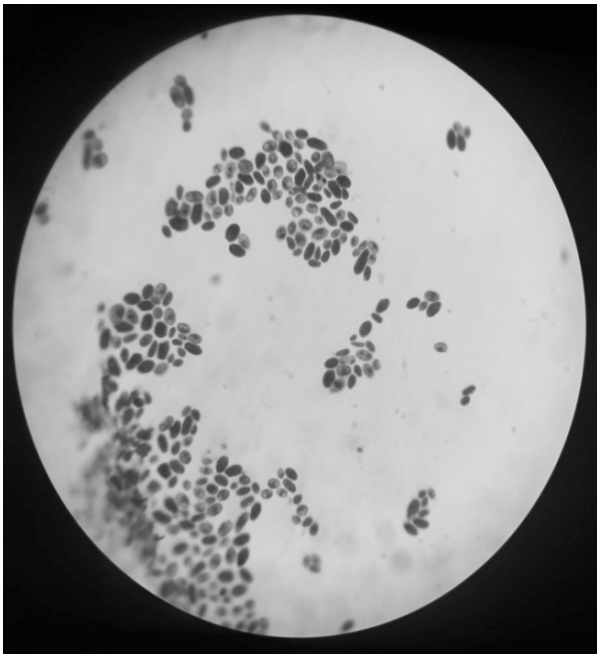

Figure 4: Yeast cells under a microscope (40x)

CONCLUSION

In this study, the presence of living lactic acid bacteria and yeasts in the kefir grain used to produce kefir was determined. In order for kefir grain to be used safely in kefir production, testing whether pathogenic bacteria are contaminated to the grain should be carried out. Furthermore, to determine which yeast and lactic acid bacteria are a presence in the kefir grain, it is necessary to isolate the species as pure, to perform biochemical tests for lactic acid bacteria, and to identify all isolates with PCR at the molecular level.

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