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Screening of Antagonistic/Synergistic Effect between Lactic Acid Bacteria (LAB) and Yeast Strains Isolated from Kefir

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***Abstract*—**Kefir is a traditional fermented refreshing beverage which is known for its valuable and beneficial properties for human health. Mainly yeast species, lactic acid bacteria (LAB) strains and fewer acetic acid bacteria strains live together in a natural matrix named "kefir grain", which is formed from various proteins and polysaccharides. Different microbial species live together in slimy kefir grain and it has been thought that synergetic effect could take place between microorganisms, which belong to different genera and species. In this research, yeast and LAB were isolated from kefir samples obtained from Uludag University Food Engineering Department. The cell morphology of isolates was screened by microscopic examination. Gram reactions of bacteria isolates were determined by Gram staining method, and as well catalase activity was examined. After observing the microscopic/morphological and physical, enzymatic properties of all isolates, they were divided into the groups as LAB and/or yeast according to their physicochemical responses to the applied examinations. As part of this research, the antagonistic/synergistic efficacy of the identified five LAB and five yeast strains to each other were determined individually by disk diffusion method. The antagonistic or synergistic effect is one of the most important properties in a co-culture system that different microorganisms are living together. The synergistic effect should be promoted, whereas the antagonistic effect is prevented to provide effective culture for fermentation of kefir. The aim of this study was to determine microbial interactions between identified yeast and LAB strains, and whether their effect is antagonistic or synergistic. Thus, if there is a strain which inhibits or retards the growth of other strains found in Kefir microflora, this circumstance shows the presence of antagonistic effect in the medium. Such negative influence should be prevented, whereas the microorganisms which have synergistic effect on each other should be promoted by combining them in kefir grain. Standardisation is the most desired property for industrial production. Each microorganism found in the microbial flora of a kefir grain should be identified individually. The members of the microbial community found in the glue-like kefir grain may be redesigned as a starter culture regarding efficacy of each microorganism to another in kefir processing. The main aim of this research was to shed light on more effective production of kefir grain and to contribute a standardisation of kefir processing in the food industry.

***Keywords*—**Antagonistic effect, kefir, lactic acid bacteria (LAB), synergistic, yeast.

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I. Introduction

KEFIR is an acidic and slightly alcoholic fermented dairy product with a distinctive taste, flavour and creamy texture. The refreshing beverage is produced by kefir grains. It contains glucose and galactose in similar amounts. Because of its biochemical properties, kefir has been used in the food industry as a thickening agent, stabilizer or emulsifier. Additionally, it has antimicrobial activity and anti-tumour activity [1]-[3]. Kefir grains are a kind of starter culture which could be defined as a gelatinous exopolysaccharide matrix having a white to yellow-white colour. They consist of embedded various LAB (10^8 CFU/g), acetic acid bacteria (10^5 CFU/g) and yeast species (10^6-10^7 CFU/g), which are defined as a symbiotic mixture of several microbial strains [3]-[6].

The kefir production process can be divided into two methods, traditional or industrial. The main difference between the two methods is a use of kefir grain or a pure starter culture for inoculation. In the food industry, standardisation of the last product is the most important demand by consumers. Therefore, in industrial plants, it is preferred to use a starter culture of kefir to achieve standardisation [7].

Each strain has a beneficial role in the kefir matrix and in the last product "kefir beverage". It is known that LAB produces lactic acid during incubation under adequate conditions, and therefore, the acidity eventually increases in the medium. Although lactic acid is important for providing the typical and distinctive flavour of the kefir beverage, in some cases its accumulation in high amounts can constitute a problem, as an increase of the acidity inhibits or retains the growth of microflora found in the kefir grain. In this circumstance, excess lactic acid accumulation should be removed from the medium. On the other hand, the organic acids produced by the natural microorganisms found in kefir prevent the growth of pathogens or spoilage microorganisms in the beverage. The concentration of acids is the most important criterion in providing the balance of advantage-disadvantage.

The microorganisms found in kefir grain are mainly composed of homofermentative and heterofermentative LAB and also lactose assimilating and non-lactose assimilating yeast strains [3], [8], [9].

Co-culture activity has been observed in kefir production. It has been known that lactose is an only carbon source found in milk. In the kefir beverage process, the lactose is fermented by LAB initially. Additionally, lactose-assimilating yeast species

found in the co-culture can degrade lactose into glucose and galactose, as well. During this period, the lactic acid increase in the medium because of lactic acid fermentation. *Saccharomyces cerevisiae*, the most common non-lactose assimilating yeast in kefir grain, can metabolize lactic acid and glucose to produce alcohol and CO_2. Thus, the symbiotic relationship is observed in between yeast and LAB [6], [8]. The efficiency of commensalism and antagonism vary by the microflora of kefir grain. In the previous studies, it was indicated that the synergistic effect was observed between non-lactose assimilating yeast and LAB. In this circumstance, it is thought that the lactic acid removal from the kefir medium is carried out by non-lactose assimilating yeast strains [8].

According to a study about the determination of the behaviours of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. cerevisiae* combination, which are two microorganisms that co-occur in Kefir fermentations, it was indicated that *Lb. delbrueckii* subsp. *bulgaricus* hydrolyzes lactose cannot be metabolized by *S. cerevisiae* into galactose and glucose. Besides, it was reported in the study that galactose could be excreted and used as a carbon source by *S. cerevisiae,* whereas it could not be metabolized by *Lb. delbrueckii* subsp. *bulgaricus*. Additionally, CO_2 accumulation is essential for growth some LAB cultures. CO_2 formation is provided by *S. cerevisiae* by alcoholic fermentation during the processes of dairy products [10].

It is known that both symbiotic/synergistic and antagonistic effects could be observed in the co-culture that consisted of yeast and LAB in kefir grain [6]. According to previous research results, it was reported that *S. cerevisiae* increases the kefiran production by promoting of *L. kefiranofaciens* which is one of a LAB culture isolated from Kefir [11]. *Kluyveromyces marxianus*, *Saccharomyces turicensis*, *Saccharomyces unispora*, *Pichia fermentans*, *Kazachastania khefir*, *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Gluconobacter frateurii*, *Acetobacter orientalis*, *Acetobacter lovaniensis*, *Weissella* sp., *Enterococcus faecalis*, *Enterococcus durans*, *Enterococcus hirae* are some yeast and bacteria strains isolated from kefir originated from different countries such as Argentina, Belgium, Brazil, Bulgaria, China, Ireland, Italy, Africa and Turkey. Although more than 20 different yeast species have been isolated from kefir, the dominant yeast species are known as *S. cerevisiae*, *S. unispora*, *Candida kefyr* and *K. marxianus* [3]. The carbohydrate sources -*maltotriose, sucrose, fructose, and glucose*- are metabolized by *S. cerevisiae* into CO_2 and alcohol mainly. These carbohydrate sources have been found in raw materials such as cereals, sugar cane, molasses, fruit, whey, etc. [12]. Alcoholic beverages have been produced since ancient times by yeast strains with their fermentative activity on carbon sources. Thus, the selection of suitable yeast strains is essential to promote the sensorial quality of beverages. The amount of acid and alcohol set the quality of kefir. Acidity is mainly originated from LAB and alcohol existence is the result of yeast fermentation. *S. cerevisiae* is the main alcohol producer in Kefir microflora [12].

Most of the time, microorganisms grown together in harsh conditions owing their resistivity and survival to the synergism between the microbial strains; this synergism could also be observed in the activity of natural extracts because their different compounds interact with each other. Thus, their activity is lesser than when these substances are together. Correlatively, the increase of microbial growth rate is determined in co-culture systems compared with the growth rate they experience alone in the medium [13], [14]. It has been reported that the kefir cultures selected in the study, *Lactococcus lactis* and *Leuconostoc mesenteroides,* as the most active polysaccharide producers when culturing them in a medium with lactose and saccharose, respectively [2].

In another study, it has been reported that probiotic properties of especially *Lactococcus* and *Lactobacillus* species isolated from kefir were determined by *in vivo* studies [15]. Research has shown that most of the microorganisms found in kefir have probiotic properties [1].

In this research, the antagonistic and synergistic effects between yeast and LAB strains were examined. Exhibiting of interaction between LAB and yeast may lead to higher productivity in the food industry. For this target, it was attempted to examine the interaction of LAB and yeast strains by cultivation experiments. Therefore, five different LAB species (*Enterococcus durans, Enterococcus hirae, Enterococcus feacium, Lactococcus lactis* and *Leuconostoc mesenteroides*) and five different strains of *S. cerevisiae* were selected among 40 strains isolated and identified from kefir samples. The effect of metabolites of LAB and *S. cerevisiae* strains in the medium were examined.

The present study was a preliminary evaluation of some LAB and *S. cerevisiae* strains isolated from kefir to find a suitable co-culture and to obtain the most effective, productive kefir grain carried out kefir beverage production. The main objectives of this study were isolation and identification of yeast and LAB cultures naturally found in kefir. Additionally, the goal of the research was the *S. cerevisiae* strains and also the antifungal effects of the selected LAB strains. As well, the synergistic effect between *S. cerevisiae* strains and LAB strains was also examined.

II. Material and Methods

A. Cultures

Yeast and LAB strains were isolated and identified from kefir samples and five strains for each bacteria and yeast group selected to determine their antagonistic/synergistic efficacy on each other. Five Gram-positive [G-(+)], catalase negative bacterial strains were isolated. They were identified as *E. durans, E. hirae, E. faecium, L. lactis* and *L. mesenteroides*. Five different *S. cerevisiae* strains were isolated and identified from kefir.

B. Isolation and Identification of LAB and Yeast Strains

Kefir samples obtained from Uludag University Food Engineering Department. For isolation; kefir samples were

diluted and spread on MRS Agar (Merck, Germany) and Malt Extract Agar (Merck, Germany) by surface plate method. The Petri dishes were incubated at 30 °C for 48 hours. Bacteria and yeast colonies were picked by a sterile needle to streak them on the individual agar for purification of isolates. After incubation, purified bacteria and yeast colonies were picked and stored in cryovials including individual broth mediums and glycerol at 30% concentration. The stock cultures in cryo-vials were stored in the freezer at -80 °C. All the strains were examined according to their macroscopic and microscopic morphological properties [16]. The bacterial strains were also examined to determine their Gram and catalase reactions. LAB and yeast cultures were identified by PCR amplification method [17]-[19].

C. Assay for Antagonistic Effect between Yeast and LAB Cultures

Antibacterial and antifungal effects were screened of *S. cerevisiae* strains and LAB strains to each other. Experiments were performed in accordance with the disk diffusion method described by [19], [20] with some modifications.

D. Determination of Antibacterial Properties of Saccharomyces cerevisiae

Antibacterial activity of *S. cerevisiae* strains was performed by the disk diffusion method. Test bacteria were grown in MRS broth medium at 30 °C for 18-24 hours. Fresh cultures were transferred onto 9 cm diameter Petri dishes containing MRS agar. Agar plates were inoculated/spread with the test microorganism (200 µL) as a thin layer that is known as the surface spreading method [19], [21]. Blank disks (Oxoid, Basingstoke, UK), 6 mm diameter, were sterilized and placed on the bacterial film. Five different strains of *S. cerevisiae* were centrifuged at 9000 rpm for 10 min and supernatants were transferred to another sterile falcon tubes. Supernatants were divided into two groups. The first group was filtered by syringe filters (ISOLAB, Sterile Syringe Filter, Hydrophobic PTFE) having 0.45 µm pore size at aseptic conditions and transferred into sterile tubes, whereas the second group was not filtered. Each blank disk was loaded with 20 µL of filtered supernatants of individual *S. cerevisiae* strains. This procedure was repeated for another group of Petri dishes containing MRS agar spread with individual LAB cultures by loading blank disks with non-filtered supernatants (20 µL) obtained from *S. cerevisiae* strains. Experiments were performed in triplicate.

E. Determination of Antifungal Properties of LAB

Antifungal activity of LAB was screened by disk diffusion method described by [19], [20] with some modifications. In this part, the test microorganisms were *S. cerevisiae* strains and blank disks were loaded with supernatants of LAB cultures individually. Experiments were performed in triplicate.

F. Assay for Synergistic Effect between Yeast and LAB Cultures

According to the literature review, it is known that synergistic effect could be observed in co-cultures like kefir grain. Because there are cell-to-cell interactions by signal molecules to promote their growth when they are together in a suitable medium and also this signalling could give microorganisms some behaviours which are not observed in mediums that cells found individually [6], [8]. In the present study, the synergistic effect was examined by plate counting method. For this purpose, *S. cerevisiae* cultures were diluted down in 9 mL sterile NaCl (0.85%) solution to reach the visible turbidity and 200 µL of each dilution was transferred to both Malt Extract Agar (MEA) and Tryptic Soy Agar (Oxoid, Basingstoke, England) and spread on the whole surface of each agar medium. Dilution and surface plate method were performed for LAB cultures on MRS Agar and Tryptic Soy Agar (TSA), as well. After the incubation at 30°C for 24-48 hours, visible colonies were observed on the surface of agar mediums. Colonies formed on the surfaces of agar mediums were counted and the rates of visible colony units were found similar on MRS Agar and TSA for LAB and on MEA and TSA for *S. cerevisiae*. In this circumstance, Tryptic Soy Broth (TSB) and Tryptic Soy Agar were the suitable mediums for the growth of both LAB cultures and *S. cerevisiae* strains.

The experiments were performed for both yeast and LAB cultures according to the plate count method on TSA described by [22] with slight modifications. All LAB and *S. cerevisiae* strains were inoculated to TSB. The treatment groups were designated as all microorganisms were inoculated both individually and two microorganisms together for each tube containing one of a LAB culture and one of *S. cerevisiae* strains. All the tubes, including TSB and test microorganisms, were incubated at 30°C for 24 hours after they were spread to the TSA by surface plate method to count the initial viable cell amounts of all experimental groups. The counting was also carried out at the 24th hour by using the same method to determine the synergistic effect between yeast and LAB cultures.

III. Results and Discussion

A. Antibacterial Properties of Saccharomyces cerevisiae

According to the disk diffusion method, zone formation was observed around the disks impregnated with neither filtered nor non-filtered supernatants of *S. cerevisiae* strains. It was observed that non-filtered supernatants had included some viable cells and they had grown around the disk. Zone formation was not observed around the visible colonies of *S. cerevisiae* strains, too. Fig. 1 represents the results of the assay for antibacterial properties of *S. cerevisiae* examined by disk diffusion method.

Fig. 2 represents one of the experimental groups and the zone formation results. These results showed that *S. cerevisiae* and LAB cultures isolated from Kefir were alive and grew in a commensal relationship.

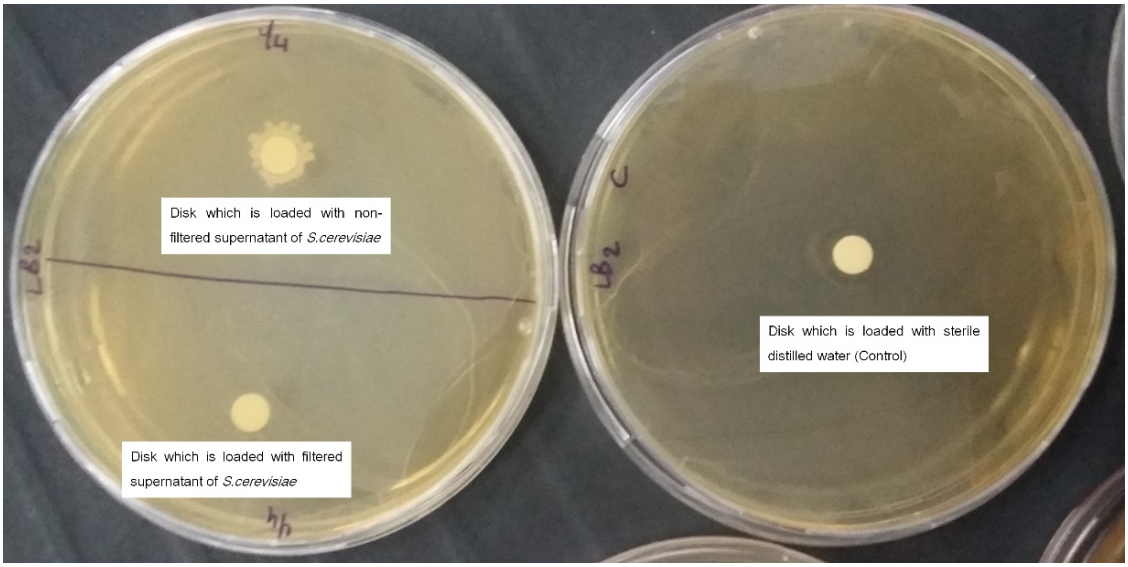

Fig. 1 The inhibition effect of filtered and non-filtered supernatants of *S. cerevisiae* strain (Y4) against *E. hirae* (LB2) isolated from the kefir sample

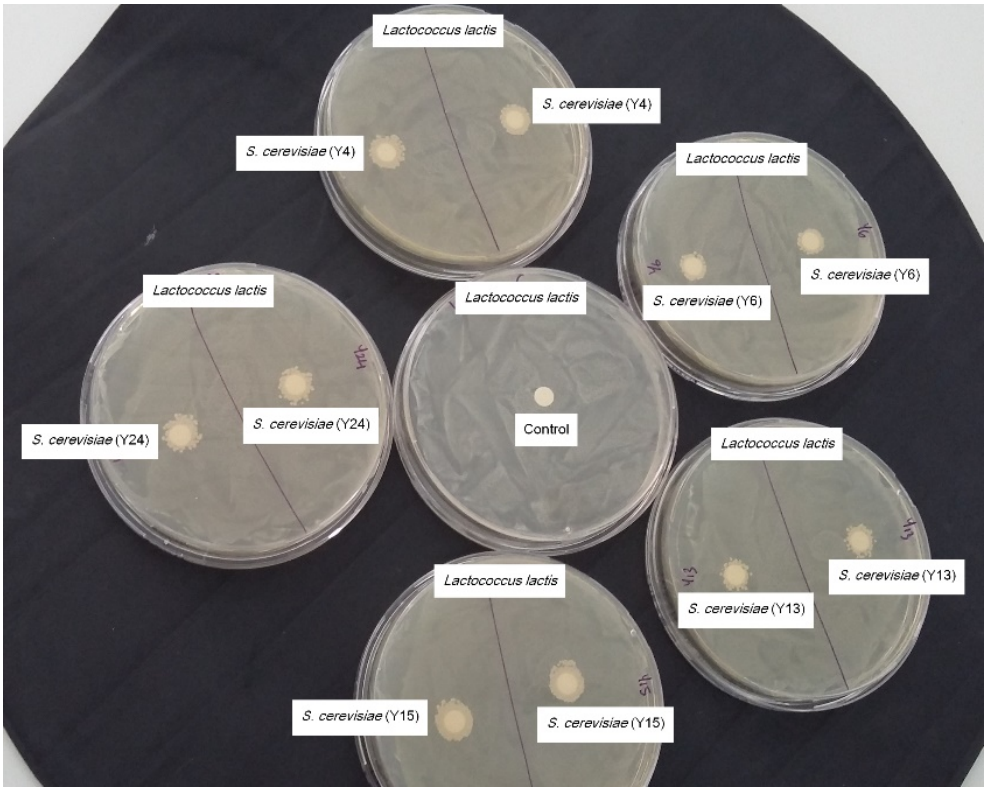

Fig. 2 The growth of five different strains of *S. cerevisiae* (Y4, Y6, Y13, Y15, Y24) and *L. lactis* (32) together in a commensal relationship

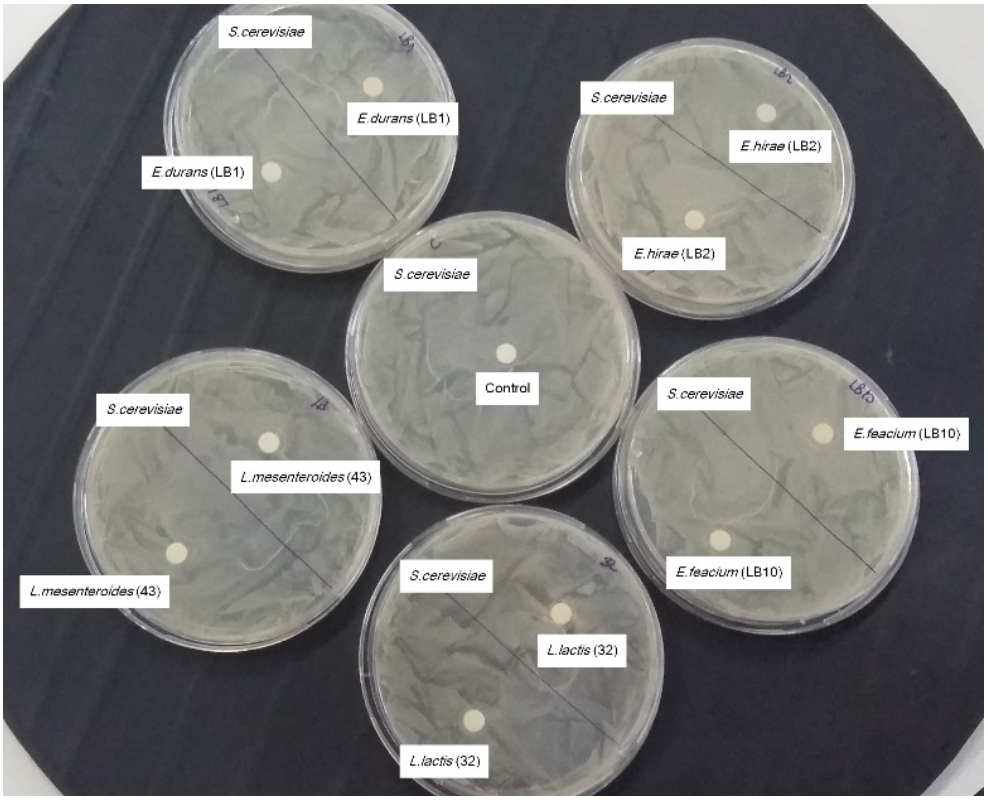

Fig. 3 The growth of five different species of LAB [*E. durans* (LB1), *E. hirae* (LB2), *E. feacium* (LB10), *L. lactis* (32), *L. mesenteroides* (43)] and *S. cerevisiae* (Y4) together in a commensal relationship

During the incubation period, CO_2 and ethanol produced by *S. cerevisiae* strains could remove from the impregnated disks with supernatants, because they are volatile compounds and are also well-known for their antibacterial efficiency. The antibacterial effect of volatile metabolites of *S. cerevisiae* was not understood within the disk diffusion method because the volatility of the compounds from the disk surface could not be prevented.

There were not any inhibition zones formed around the disks loaded with supernatants of *S. cerevisiae*, and thus, the result demonstrated that metabolites other than CO_2 and ethanol had no inhibitory effect on LAB cultures.

B. Antifungal Properties of LAB

According to the disk diffusion method, zone formation was not observed around the disks placed on the TSA that inoculated with *S. cerevisiae* strains (Y4, Y6, Y13, Y15, Y24) by surface plate method and disks were impregnated with supernatants of *E. durans, E. hirae, E. feacium, L. lactis, and L. mesenteroides.* Inhibition zones were formed around the disks loaded with neither filtered nor non-filtered supernatants. Fig. 3 represents the commensal relationship between different species.

Commensalism was observed in the Petri dishes. LAB metabolize the carbon sources and produce lactic acid mainly. Lactic acid is one of the non-volatile organic acids; thus, the results indicated that lactic acid accumulation was not enough to inhibit the *S. cerevisiae* strains selected in the present study. The lactic acid fermentation could be carried out by hetero- and/or homo-fermentative LAB. The last products change according to the fermentation method.

Acetic acid and CO_2 are well-known volatile compounds which are produced during lactic acid fermentation [23], [24]. Acetic acid is produced in very small amounts as compared with lactic acid; thus, its inhibition effect has not big importance in the medium. CO_2 accumulation in the medium is more effective to inhibit some microorganisms. In this study, the results indicated that metabolites formed during the incubation except for CO_2 and acetic acid had no inhibitory effect on selected *S. cerevisiae* strains.

C. Synergistic Effect between Yeast and LAB Cultures

In this experiment, symbiotic/synergistic effects were observed for some cultures grown together on TSA. Bacteria and yeast colonies were counted on the agar medium where they were grown singly and together with each other. According to the counting results, *S. cerevisiae* (Y24) strain promoted the growth of *L. lactis* (32) as it is seen in Table I.

The amount of *L. lactis* increased 2.4 log units when it has grown on TSA singly, whereas it increased about 3.0 log units when it has grown on the same agar medium with *S. cerevisiae* (Y24). Also, some LAB cultures promoted the growth of yeast cultures. *S. cerevisiae* (Y6) had an increase in 2.4 log unit when it was incubated alone at 30°C for 24h. A

slight increase (3.0 log units) of yeast culture growth was observed when it was grown with LB2 strain, *E. hirae*.

The same increase in growth rate was observed in Y13 strain of *S. cerevisiae* when it was grown on TSA with *E. hirae*, as well. Furthermore, counting results shows that *E. hirae* induced the growth of another strain of *S. cerevisiae* (Y24). The most promoting effect was observed in one of the experimental groups which *L. mesenteroides* (43) and *S. cerevisiae* (Y24) grown together on TSA. The number of viable cells of Y24 increased in 2.6 log units when it was grown singly, whereas the sharp increase, about 4.0 log units, was determined when it has grown together with *L. mesenteroides.* All the logarithmic increases of microbial growths were shown in Table I.

TABLE I
MICROBIAL LOADS OF EXPERIMENTAL GROUPS AT THE BEGINNING OF AND AFTER THE INCUBATION

Experimental groups	**Microbial count at 0. hour**		**Microbial count at 24. hour**	
	LAB log (cfu/mL)	Yeast log (cfu/mL)	LAB log (cfu/mL)	Yeast log (cfu/mL)
LB1	6.9	0	8.7	0
LB2	6.9	0	8.7	0
LB10	6.7	0	8.8	0
32	6.4	0	8.8	0
43	5.5	0	7.5	0
Y4	0	4.3	0	6.6
Y6	0	4.0	0	6.4
Y13	0	4.3	0	6.7
Y15	0	4.2	0	6.8
Y24	0	4.2	0	6.8
LB1+Y4	6.8	4.7	8.1	6.8
LB1+Y6	6.3	3.9	8.0	6.2
LB1+Y13	6.7	4.7	8.4	6.2
LB1+Y15	6.8	5.1	8.4	6.4
LB1+Y24	6.8	5.3	9.0	6.8
LB2+Y4	6.4	4.8	8.8	7.0
LB2+Y6	6.9	**4.5**	8.5	**7.5**
LB2+Y13	6.8	**5.0**	8.7	**8.0**
LB2+Y15	6.1	5.2	8.5	7.7
LB2+Y24	6.8	**4.8**	8.6	**8.0**
LB10+Y4	6.8	5.1	8.3	7.2
LB10+Y6	5.6	4.6	8.3	6.6
LB10+Y13	6.7	5.3	8.6	7.0
LB10+Y15	6.8	4.5	8.5	6.7
LB10+Y24	6.7	5.0	8.5	6.6
32+Y4	6.5	5.0	8.6	7.6
32+Y6	6.6	4.4	8.5	5.2
32+Y13	6.6	5.2	8.2	6.0
32+Y15	6.6	4.6	8.5	6.6
32+Y24	**6.7**	5.1	**9.5**	7.0
43+Y4	5.7	4.4	7.5	6.7
43+Y6	5.3	4.7	6.8	6.6
43+Y13	5.6	4.8	6.7	6.6
43+Y15	5.8	4.2	6.6	6.5
43+Y24	5.7	**4.4**	8.6	**8.3**

LB1: *Enterococcus durans*; LB2: *Enterococcus hirae*; LB10: *Enterococcus feacium*; 32: *Lactococcus lactis*; 43: *Leuconostoc mesenteroides*; "Y4, Y6, Y13, Y15, Y24": *Saccharomyces cerevisiae*

Although disk diffusion method demonstrated that there were not any inhibition effect of metabolites produced by LAB and *S. cerevisiae* strains against each other, the efficiency of volatile compounds occurred during incubation period could not be understood by disk diffusion method. However, the experiment, which was carried out to determine the synergistic effect between cultures, also showed the inhibitory efficiency of volatile compounds produced in broth medium.

In the study, screw-capped tubes were used for keeping sterile TSB and all test microorganisms inoculated in. Thus, volatile and non-volatile compounds could be kept in the broth medium during the incubation period. After 24 hours, small amounts of all experimental groups were transferred to TSA to count viable cells after incubation. According to the counting of visible colonies on agar medium demonstrated that neither non-volatile nor volatile compounds produced by both LAB and *S. cerevisiae* strains had the inhibitory effect on each other. Additionally, some of them promoted the growth of another microorganism that they formed as co-culture together.

IV. CONCLUSION

The use of mixed cultures in fermentation processes may provide the metabolic pathway for the utilization of complex compounds and may promote the microorganisms to produce organic components which could be an enhancer for the growth of each other [25]. In mixed cultures, both antagonism and synergism are observed naturally. This balance promotes them to survive together.

In the present study, it was observed synergistic effect and commensalism among the yeast and LAB strains isolated from kefir sample. It is well known that *S. cerevisiae* possesses catalase activity. Thus, the selected yeast strains might reduce the amount of hydrogen peroxide (H_2O_2) which is produced by some LAB. The accumulation of H_2O_2 in the medium could cause self-growth-inhibition effect, and therefore, LAB could be inhibited by its own metabolites. *S. cerevisiae* can remove H_2O_2 by catalase activity and can also reduce the concentration of lactic acid produced by LAB in mixed culture.

According to the results of the present study, it has been thought that metabolites of one microorganism could induce growth of another microbial strain in the medium. On the other hand, the results showed that test microorganisms shared the energy sources to survive in the medium without inhibiting each other. Furthermore, some metabolites of a microorganism could be a carbon or energy sources of another microorganism. In this circumstance, a commensal relationship may appear by growth in co-culture.

All of the members of the microbial community found in kefir grain should be identified and examined to understand the relationship between them. Thus, studies may shed light on the production of more effective kefir grain and to contribute to standardisation of kefir processing in the food industry.

In this study, single and couple behaviours of test microorganisms were researched. Following, studies should be

planned to determine the behaviours of microorganisms in the medium which included more than two cultures grown together.

It has been thought that all the experiments may provide a basis to build the new co-culture composed enhancer microorganisms isolated from kefir. Thus, a standardised refreshing kefir beverage can be obtained by using more effective strains found in kefir grain naturally. The effective mix culture can promote the production rate of the beverage in the food industry.

Most the microorganisms found in natural kefir microbiota are well-known as probiotic microorganisms which are beneficial to human health. The next step in future studies may be a selection of distinctive microbial strains and the formation of more effective industrial kefir grains containing more probiotic bacteria to enhance the human immune system.

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