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Characterization of Antimicrobial Peptide Fraction Producing *Lactobacillus* spp. Based on LC/MS-MS and Determination of ACE-inhibitory Activity in Kefir

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ABSTRACT
In the present study, bioactive properties such as ACE-I activity and antimicrobial activity of kefir using different *Lactobacillus (Lactobacillus delbrueckii ssp. bulgaricus* ATCC 11842, *Lactobacillus helveticus* ATCC 15009 and *Lactobacillus plantarum* ATCC 14917) on some pathogen and Gram-positive bacteria during 28 day-storage periods was investigated and proteolysis (RP-HPLC peptide profiles, RP-HPLC amino acid profiles) were studied. The antimicrobial activity was investigated in kefir extract and separated peptide fractions (<3 kDa, named F2) which were characterized by LC-MS/MS and precursor and product ions were determined. Antimicrobial activity has been observed against pathogenic bacteria such as *Escherichia coli (E. coli)* in all samples. But the results revealed that the F2 fraction separated from kefir manufactured using Lactobacillus had a stronger antibacterial effect than control samples. It was determined that the F2 fraction has antimicrobial activity against *S. aureus*, *S. warneri* 95052 and *S. hominis*. The ACE-I activity of samples A, B, C and K were 76.47%, 84.95%, 87.33% and 85.57%, respectively. In the kefir using *Lactobacillus* has increase of ACE-I activity and was significant (P<0.01). In this study, it was concluded that the using of adjunct *Lactobacillus* contributed to the functional value of kefir.

Keywords: Bioactive peptides, In-Vitro antimicrobial peptides, ACE- I activity, purification, LC-MS/MS

1. Introduction

Kefir is a fermented dairy beverage produced by the actions of the microflora encased in the "kefir grain", the composition differing according to the source, are a symbiotic association of a variety of bacteria and fungi, such as lactic acid bacteria, acetic acid bacteria, yeasts and molds, etc., originated thousands of years ago in the Caucasus (Pogačić et al. 2013; Purutoglu et al. 2020; Wang et al. 2021). In kefir, the predominant genus was *Lactobacillus*. Low abundant genera, such as *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Acetobacter*, etc were also found from different grains. Yeasts were abundant in fungal microbiota of kefir grains and genera belonging to *Saccharomyces*, *Kazachstania*, *Kluyveromyces*, *Pichia*, etc. (Tas et al. 2012; Prado et al. 2015; Garofalo et al. 2015). The microbiota of kefir grains has been found depending several factors such as the origin of the kefir grain, grain cultivation methods, sanitation conditions, production and storage conditions (Witthuhn et al. 2004; Gul et al. 2018). Accordingly, the differences of microbiota in kefir grains can be responsible for important changes in nutritional and flavor properties of kefir product. It has been determined that milk proteins have the physiological activities by some peptides that are digested with gastric and pancreatic enzymes (Schanbacher et al. 1998). Peptides resulting from proteolysis by microorganisms in kefir grains partially responsible for the biological activity of kefir. Bioactive peptides have been proposed as health-promoting compounds and these peptides comprising 2 to 20 amino acids residues with functional and biological properties such as antihypertensive, antioxidant, antimicrobial, anticancer and opioid (Karami & Akbari-Adergani 2019; Zhu et al. 2019). Many of studies have investigated production and activity of bioactive peptides, i.e. antioxidants, antimicrobial, antithrombotic and immunomodulatory activities (Chandra & Viji 2018; Chandra et al. 2019). Among these peptides, one of the most important and widely studied is ACE-I peptides. ACE is an exopeptidase and it elicits dipeptides from the C-terminal ends of various peptide substrates (Pihlanto-Leppälä 2000). Bioactive compound production may be strikingly increased by controlling the fermentation conditions (Zajsek & Gorsek 2011). Either as starter, as adjunct cultures, or as probiotics, lactobacillus strains are used as food preservatives not only to prevent the development of food spoilage but also to give consumers a health benefit. Some lactobacilli produce bacteriocins, proteins active against other bacteria. According to the literature is examined, it has been found that lactic acid bacteria such as *L. helveticus*, *L. casei* are extensively used in the fermentation of some dairy products (Nielsen et al. 2009; Otte et al. 2011; Sanlı et al. 2018). In a study, it was reported that the antihypertensive peptides can be produced during fermentation of lactic acid bacteria used as adjunct cultures in kefir production contributed in different ways to peptides and also slightly contributed to the formation of ACE-I peptides (Sanlı et al. 2018). However, only a few studies have been published on the ACE-I activity of kefir (Maeda et al. 2004; Quiros et al. 2005) and it needs to be further studied. The types and strains of starter microorganisms used in kefir have affected the bioactivity of the product. The data obtained in a

study showed that the variability in the amino acid content of kefir is a function of the culture type (Ozcan et al. 2019). It was determined that the principal amino acids of buffalo milk kefir produced either by grains or adjunct starter culture were glutamic acid, alanine, serine, tyrosine and the like (Ozcan et al. 2019). Bioactive peptides originating from milk proteins were identified in amino acid sequences of these proteins. It has been shown that these peptides can be expressed by bacterial microbial, coagulant, digestive enzymes, and exogenously added starter lactic acid bacteria (Gobbetti et al. 2002). For this reason, functional foods and bioactive components have begun to attract the attention of food scientists, nutritionists, health professionals and consumers. In a study, it was reported that kefir enriched in terms of probiotic microorganisms was equivalent to traditionally produced kefir, its bioavailability and functionality has been increased, and it was suitable for industrial production and likeable regarding organoleptic properties. (Karacalı et al. 2018). Furthermore, kefir represents a good choice as a probiotic food carrier, showing potential advantages for human health over other dairy fermented products. Foodborne diseases, which can cause more than 900 million infectious events, have become a public health problem (He et al. 2018). These diseases are caused by eating foods contaminated with pathogenic microorganisms such as *S. aureus* and *E. coli*. Although the growth of pathogenic bacteria in foods can be effectively controlled with chemical preservatives, consumers are still concerned about some safety issues (Ma et al. 2020). In addition, increased bacterial resistance to common and artificial antibiotics and their side effects have also caused problems in public health leading to the discovery of natural antimicrobial counterparts (Pina-Perez et al. 2015; Sundin & Wang 2018). In addition to its antibacterial, antifungal and antitumor activities, kefir is effective in improving health, strengthening the immune system, balancing blood pressure and lowering serum cholesterol levels. (Ajam & Koohsari 2020). Therefore, there is a need for the development of natural antibacterial agents. Recently, antimicrobial peptides (AMPs) have been gaining widespread attention due to their excellent functional activities. Many natural AMPs such as nisin have been reported today (Miao et al. 2016). However, nisin is the only antimicrobial peptide used in the food field as a natural food preservative (Upendra et al. 2016). Therefore, AMPs are of great importance as a potential food preservative. Several studies are investigating the antibacterial activity of kefir. However, little information is readily available concerning the characterization of antimicrobial peptides from kefir produced using *Lactobacillus*.

This study aimed to the effect of ACE-I activity and the antibacterial activity against selected bacteria in kefir samples produced using *Lactobacillus*. Also, the results can provide information on the amino acid content, to enable a better understanding of the ACE-I activity of kefir. Additionally, characterization of AMP peptide fractions by Liquid chromatography-Mass spectrometry-Mass spectrometry (LC-MS/MS) was provided.

2. Material and Methods

2.1. Materials

Kefir grains were purchased from a local market and groved in skim milk at 22 °C for 24 hours a day. The kefir granules were obtained by continuously growing in skimmed milk. Kefir grains, raw cow's milk used in the production of kefir samples and *Lactobacillus delbrueckii* ssp. *bulgaricus* (*L. delbrueckii* ssp. *bulgaricus*) ATCC 11842, *Lactobacillus helveticus* (*L. helveticus*) ATCC 15009, *Lactobacillus plantarum* (*L. plantarum*) ATCC 14917 used adjunct cultures were provided from the Food Engineering of Inonu University Engineering Faculty (Malatya, Turkey). Raw cow's milk was heated 55-60 °C, then fat value was standardized to 2% with a cream separator. After pasteurization (90 °C for 5 min), the milk was cold at inoculation temperature (23 ºC) and is divided into 2 parts. The first part of milk at aseptic conditions, 3% kefir grains are inoculated and incubation is continued for approximately 22-24 hours at 20-25 ºC. When the curd acidity reaches pH 4.6, the incubation process was terminated and the kefir grains were separated by filtration (sample K). Second part of milk was inoculated kefir grains at level of 3% (w/v) and the incubation was continued for approximately 22-24 hours at 20-25 ºC until pH 4.8, the kefir grains were separated by filtration. The fermented milk divided into 3 parts. Each part was inoculated with the adjunct starter culture (sample A: *L. delbrueckii* ssp. *bulgaricus*, sample B: *L. helceticus*, sample C: *L. plantarum*, 1mL per 100 mL). When the second incubation reaches pH 4.6 at 37 ºC, the incubation process was terminated. Kefir samples are stored in 200 mL plastic containers at 4 ºC during 28 days.

A 20 g of Kefir sample was mixed 10 mL deionized water and incubated in a shaking water bath for 1 hour at 40±1 °C. Kefir was centrifuged at 3000 × *g* for 30 min at 4 ºC with a cooled centrifuge (model 320R, Hettich, Tutlingen, Germany). Supernatants were filtered through qualitative filter paper (Whatman No: 113) The water-soluble nitrogen (WSN) fractions were freeze-dried used to HPLC peptides analysis and ACE-I activity analysis and antimicrobial activity of the kefir samples.

2.2. Proteolysis

Peptide profile of the freeze-dried WSN fractions of the kefirs was analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) (Shimadzu LC 20 AD Prominence HPLC) system (Shimadzu Corporation, Kyoto, Japan) according to the method described by Sulejmani & Hayaloglu (2017). The RP-HPLC individual free amino acids contents of water-soluble fractions were determined as previously described by Hayaloglu (2007). The total Free Amino Acids (FAAs) content of the kefir samples was determined by the method described in Sahingil et al. (2019).

2.3. RP-HPLC determination of angiotensin-converting enzyme activity

The ACE-I activity and IC_{50} value were determined as previously described by Sahingil et al. (2019).

$$IC_{50} = \frac{BSAx50}{IR}$$

BSA (Bovine Serum Albumine): mg/L; *IR*: ACE inhibition rate (%)

2.4. Antimicrobial activity

The antimicrobial activity assay was studied in both kefir and WSN fractions obtained from kefir. The kefir was centrifuged at 3000 × *g* for 30 min and the supernatant passed through 0.45 µm pore size filter for sterilized. The supernatans were kept at refrigerator temperature until antibacterial activity tests. WSN fractions were freze-dried used for antimicrobial activity tests. The dried water-soluble fractions were dissolved in ultrapure water to 50 mg/mL. The fractions were separated according to their molecular size using centrifugal filters (Amicon Ultracel-3K, Merck-Millipore Ltd, Cork, Ireland). Then the supernatant was sterilized by filtration using a 0.45-µm pore size syringe filter. *Staphylococcus epidermidis* RSKK 0802 (*S. epidermidis*), *Staphylococcus aureus* 1021/06008 (*S. aureus*), *Staphylococcus warneri* 95052 (*S.warneri*), *Staphylococcus xylosus (S. xylosus), Shigella flexneri* RSKK 184 (*S. flexneri*), *Bacillus cereus* RS 863 (*B. cereus*), *Escherichia coli (E. coli)* (Microbiology laboratory of Inonu University Turgut Özal Medical Center), *Enterococcus faecalis (E. fecalis), Candida albicans* ATCC 04055 (*C.albicans*), *Streptococcus mutans (Str.mutans), Enterobacter, Salmonella enteritidis (S.enteritidis), Pseudomonas putida* (*P. putida*) were investigated on antimicrobial effect on 13 different microorganisms. They were procured from Inonu University. Then, the bacteria were recovered in the Brain Heart Infusion medium (Merck) at 37 ºC/24 hr in the microbiology laboratory of the Inonu University. Antimicrobial inhibition effects on some pathogenic bacteria and yeast were determined by disc diffusion method (0.6 mm disc diameter). The macrodilution tube method was used based on turbidimetric assay and the highest inhibition zone diameter was selected for the minimum inhibitory concentration (MIC) of kefir. For WSN fractions 5 different concentrations (50-200 mg/mL; 50-75-100-150-200 mg/mL) which prepared in Nutrient Broth (Merck) were studied for determination of minimum inhibitory concentration. The dilution (100 mg/mL) in which microbial turbidity was not observed, as the MIC was reported. A hundred microliters of each WSN extract were by disk diffusion method onto the surface of the agar. For kefir extract 10 different concentrations (100-1000 µL/mL; 100-200-300-400-500-600-700-800-900-1000 µL/mL) were studied for determination of minimum inhibitory concentration. The dilution (1000 µL/mL) in which microbial turbidity was not observed, as the MIC was reported. The plates were incubated for 24 h at 37 ºC in anaerobic media. The lyophilized peptide fractions (100 mg/mL) were dissolved in 40 mM sodium phosphate buffer (pH 6.5) and incubated for 24 hours at 37 ºC against the test microorganisms. Subsequently, the susceptibility and resistance of each bacteria were determined by measuring the inhibition zone diameter (IZ) (0.6 mm disc diameter) around the wells. The antibiotic standard (Sigma-Oxytetracycline) was used to compare the results (10 mg/mL antibiotic 100 µL enject); 100 mg/mL- dry extract 100 µL, 100 µL/mL- kefir extract 100 µL.

2.5. Isolation of antimicrobial peptides for LC-MS/MS in RP-HPLC

Water-soluble fractions were separated according to their molecular size using centrifugal filters (Amicon Ultracel-3K, Merck-Millipore Ltd, Cork, Ireland). Since peptides showing bioactive properties are generally peptides containing 2-20 amino acids, peptide profile of peptides smaller than 3 kDa was determined by RP-HPLC analysis and they were collected into separate fractions. The total peptide analysis for 80 minutes for fractionation is given in Figure 1b. Antimicrobial activity was examined in the fractions separated from the fraction collector. The peptide fraction named F2 was determined to have antimicrobial ctivity.

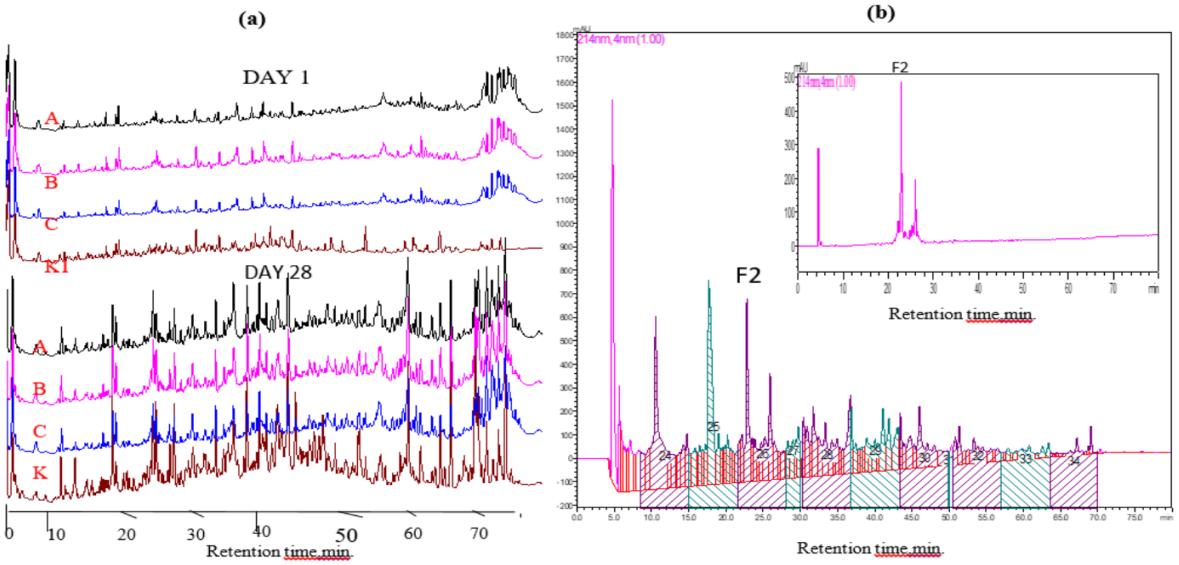


Figure 1- (a) RP-HPLC peptide profile of the kefir samples. Sample K, kefir granule containing; sample A: kefir granule+ *L. bulgaricus*; sample B: kefir granule+ *L. helveticus*; sample C: kefir granule+ *L. plantarum*. (Day 1, 28) (b) Separating peptide fractions of kefir that are less than 3 kDa in HPLC fraction collector. F0, 8.50-15.17 min; F1, 15.20-21.60; F2, 21.70-28.18 min; F3, 28.22-30.00 min; F4, 30.25-36-82 min; F5, 36.83-43.38 min; F6, 43.40-49.88 min; F8-10, 50.45-70.00 min.

2.6. Characterization of antimicrobial peptides in LC-MS/MS

Fractions were separated as shown in Figure 1b and the F2 fraction with antimicrobial activity was analyzed under the conditions specified using liquid chromatography-mass spectrometry (LC-MS/MS) system (Shımadzu LC-MS 8030, Japan) with 0.1% formic acid in 2% acetonitrile as the eluent. Fractions F2 was separated on a C18 reverse-phase capillary column (Agilent Technologies, Zorbax column, 4,6×250mm×5um). It was column oven 40 ºC, 15 µL sample was injected and a flow was set at 1 mL/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in water) from 5 to 95% in 70 min. The mass spectrometer was operated in the positive mode with a nebulizer pressure of max 60 MPa. Peptide analysis was performed using data-dependent acquisition of one MS scan (scan range from 100 to 1 900 m/z, depending on the m/z precursor ion) followed by a tandem MS (MS/MS) scan of the 5 most abundant ions in each MS scan.

2.7. Statistical analysis

An ANOVA followed by Duncan test was performed with a 95% confidence, using the SPSS program (SPSS package program, version 13.0, SPSS Inc., USA). Data obtained from two trials were analyzed in duplicate, microbiological analyzes were analyzed in triplicate. Principal component analysis (PCA) was performed using a covariance matrix and varimax rotation between the kefirs (version 9.0, SPSS Inc., Chicago, IL). The results obtained were directly taken from automatic reporting in LCMS Lab solution Ver 5.72 without any further data manipulation except for the retention time adjustment and peak area integration review. For experimental parameters used the Class-Agent authentication database.

3. Results and Discussion

3.1. The RP-HPLC peptide profiles of the kefirs

RP-HPLC peptide profiles of days 1 and 28 of the Kefir samples are shown in Figure 1a. The RP-HPLC peptide profiles of the kefirs were precise and no significant discrepancy between the kefirs was detected. On the 28th day of storage in sample K, it was found out that almost no new peptides were reproduced with the except of certain ones. After the 28th day of storage, presence of some peptides was detected in the sample K at the highest concentration point. However, some minor differences were observed depending on the storage period in RP-HPLC chromatograms of kefir samples. During storage, almost identical peptide profiles were observed in all the kefir samples. Peptide profiles of samples A, B and C were similar on all days of storage except for sample K (Figure 1a).

3.2. The RP-HPLC free amino acid profiles of the kefirs

RP-HPLC free amino acids profile and PCA diagram of RP-HPLC free amino acids of the kefir samples were given (Figure 2a and Table 1) at 1 and 28 days of storage. The level of free amino acids such as Gln, Gly, Thr, Asp, and Ser was higher in the control sample compared to other kefir samples. It was observed that the amount of Arg, Glu and Pro increased in at the end of the storage period. The content of Trp, Asn, Ala, Gly increased in samples B and K during storage. Val content decreased during the storage period while Ile content increased in samples B, C and K. Amino acids at the highest concentration in sample K (control) were Ala, Glu, Gly, Asp, Phe respectively while in kefir with adjunct lactobacilli were Ala, Pro, Arg, Leu, Lys. Glu and Lys as an antioxidative component were found (9.39 for K, 8.87 mg/100 g for B) to be present at a high concentration. It has been reported that the intensity of the scavenging activity of peptides could be affected by the hydrophobic amino acid number. In a study, the content of hydrophobic amino acids such as Lys, His and Arg contributed to Fe^{2+} chelating activity, while Met, Pro, Cys, Ala, Gly, Val and Leu have a higher radical scavenging ability (Zhu et al. 2013). The presence of lysine is believed to increase the antioxidant capacity of peptides (Huang et al. 2017). The other study was identified the antioxidative peptides included Glu and Lys amino acids in goat milk fermented by *L. plantarum* 60 (Chen et al. 2021). The added adjunct cultures have a pronounced effect on the free amino acid content of kefir samples. The Pro concentration had the highest value in sample C (*L. plantarum*), followed by sample A (*L. delbrueckii* subsp. *bulgaricus*) and sample B (*L. helveticus*). The presence of Pro, Lys and Arg amino acids at the C-terminal ends of ACE-I peptides is generally indicated to increase their bioactivity. Pan et al. (2005) found that antihypertensive peptide was formed in skimmed milk fermented with *Lb. helveticus* JCM1004 and that VPP and IPP peptides including proline had an effect on ACE-I activity. It is suggested that the bioactivity of antihypertensive peptides is due to the hydrophobicity and positively charged amino acids of the peptides in the structure, which are generally found (Pripp et al. 2004). Proline found the highest amount in the free amino acid composition of kefir samples formed of kefir grains applied to conventional milk (Ultra High Temperature) and certificated organic milk, while Ala, Asp, Lys, Arg and Cys is followed by Güler et al. (2016). It was reported that the presence of Try, Val, Lys, Met, Phe, Thr and Ile in kefir (Liutkevičius & Šarkinas, 2004). In a study investigating the chemical properties of Norwegian kefir, it was stated that Glutamic acid has the highest value among free amino acids (Grønnevik et al. 2011). Proline and Glutamic acid had the highest amount of free amino acids; Alanine was the second most abundant amino acid in kefir produced using *L. bulgaricus* HP1 and *L. helveticus* MP12 (Simova et al. 2002). Leucine is classified as an essential amino acid that gives chance to determine the degree of proteolysis. Leu was found at the lowest amount in sample K, the highest values of these amino acids were B added *L. helveticus*. In a study, it was reported that kefir sample supplemented *L. helveticus* MP12 which has high peptidase and aminopeptidase activity compared to *Lactococcus* species, have a unique amino acid profile as respect to control samples, and Leu, Ile, Val, Lys, Phe and Met levels

were found to be 1.5 times higher (Simova et al. 2006). Enzyme systems with different culture types and proteolytic activities cause the degradation of peptides and result in different kinds and amounts of amino acids. Similarity to this study, it was reported that the principal amino acids of kefir produced either by grains or commercial kefir culture including lactobacillus culture were glutamic acid, alanine, proline, valine and the like (Gul et al. 2018; Ozcan et al. 2019). The results of the free amino acid analysis of the kefirs were subjected to basic component analysis (PCA) and the graph showing the results of the analysis is shown in Figure 2a). In the PCA graph, which analyzed the results of the storage on days 1, 7, 14, 21 and 28, it was determined that kefir had a distribution depending on the storage period, the addition of *Lactobacillus*, and that kefirs exhibited a different amino acid profile. It was found that *L. plantarum* was separated from the other kefir samples (circled in Figure 2a) and contained a higher concentration of amino acids than other kefir samples. Supplementation of *L. helveticus* H9 enhanced fermented milk acidification and proteolysis. These bacteria generally have high extracellular proteinase activities and thus release specific bioactive peptides during milk fermentation (Nielsen et al. 2009; Zhou et al. 2019). *L. helveticus* is known to have high proteolytic activity (Ahtesh et al. 2017). Proteolytic activity of *L. plantarum* and *L. helveticus* strain were evaluated by Beganović et al. (2013). The remarkable proteolytic activity of these strains is possibly due to their ability to release extracellular and intracellular proteases during fermentation (Indarmawan et al. 2016; Fang et al. 2018). The total FAAs levels increased due to storage period (Figure 2b). The use of *Lactobacillus* as an additional culture resulted in a significant increase in total FAAs levels due to proteolytic activity. It was determined that the kefir produced by *Lactobacillus* (*Lb. plantarum* and *L. helveticus*) adjunct starter has a total FAAs value that is higher than the control samples at the end of storage period.

Table 1- HPLC-Free amino acids of kefir samples (mg/100g kefir) storage at 4 °C in 1 and 28 days

	DAY 1				*DAY 28*				*Statistics*	
Amino acids	*A*	*B*	*C*	*K*	*A*	*B*	*C*	*K*	P_d	P_s
ASP	1.76	1.65	2.7	1.6	7.04	5.46	5.36	8.35	***	***
GLU	2.65	1.38	2.57	9.65	7.53	7.18	9.15	9.39	***	***
ASN	0.07	0.96	0.6	0.5	0.95	4.01	0.79	0.97	**	***
SER	0.4	0.35	2.69	3.08	3.8	0.33	4.45	5.08	***	***
GLN	0.05	0.19	0.48	0.39	0.87	0.24	0.76	0.96	***	***
GLY	0.24	1.99	2.04	1.84	5.67	6.06	7.14	9.09	***	***
HIS	1.34	6.18	5.62	6.48	5.74	5.18	7.85	6.16	***	***
ARG	1.11	2.41	2.39	2.06	8.54	3.14	11.81	1.46	***	***
THR	3.63	1.67	2.61	1.85	4.37	2.08	2.3	1.89	***	***
ALA	11.67	16.18	12.85	11.8	16.13	16.98	16.31	12.66	***	***
PRO	5.51	2.95	4.75	3.93	7.7	5.58	12.35	4.89	***	***
TYR	3.77	2.18	3.33	2.63	3.02	4.45	5.7	2.22	***	***
VAL	6.35	5.92	6.86	5.46	4.32	2.2	0.62	2.39	***	***
MET	0.65	1.69	0.21	1.84	6.1	4.76	2.15	3.72	***	***
CYS	1.1	1.55	5.61	2.67	3.84	5.93	10.84	7.03	***	***
ILE	0.45	0.4	0.6	0.38	0.57	4.97	2.31	3.32	***	***
LEU	4.46	8.54	7.51	7.27	7.66	9.7	8.22	7.21	***	***
PHE	0.33	5.97	2.76	6.79	7.41	6.14	3.22	7.38	***	***
TRP	2.94	1.16	1.12	1.89	6.41	4.13	1.84	5.15	***	***
LYS	2.41	4.53	5.67	4.01	3.21	8.87	7.07	6.41	***	***
TOTAL	50.88	67.83	72.97	76.11	110.88	107.4	120.24	105.73	***	***

P probability; * P<0.05; ** P<0.01; *** P<0.001; P_d: Day; P_s: sample; sample K, kefir granule containing; sample A: kefir granule+ *Lb. bulgaricus*; sample B: kefir granule+ *Lb. helveticus*; sample C: kefir granule+ *Lb. plantarum*.

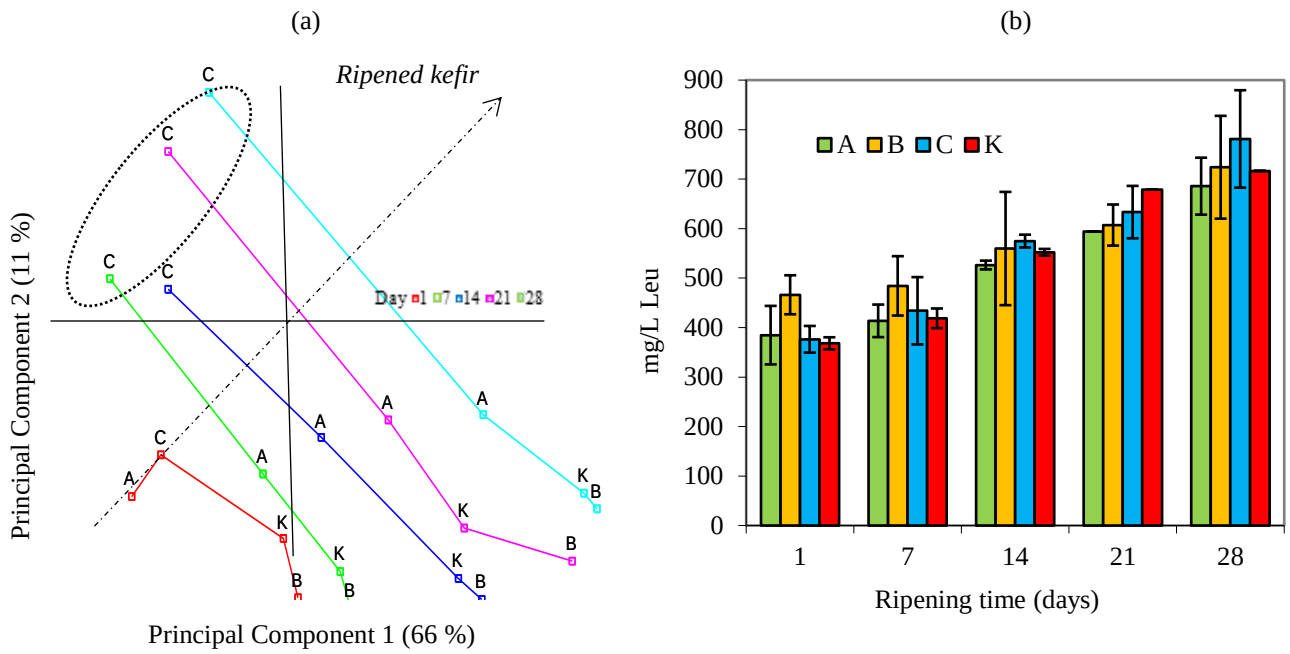


Figure 2- (a) PCA diagram of RP-HPLC Free Amino Acids from samples. Sample K, kefir granule containing; sample A: kefir granule + *L. bulgaricus*; sample B: kefir granule + *L. helveticus*; sample C: kefir granule + *L. plantarum*. (b) Total free amino acids of kefir samples (mg/L, Leu)

3.3. ACE-I activities of kefir samples

Fermented dairy products, such as yogurt, different types of cheese and kefir, contain peptides with ACE-I activity, these inhibitors are used to cure high blood pressure and hypertension (Shu et al. 2017). Proteins are hydrolyzed into plenty of bioactive peptides, antihypertensive activity, by lactic acid bacteria during fermentation especially lactobacilli is one of its important bio-functional properties. It was also found that ACE-I activity as IC_{50} was significantly increased in sample C (14.84±1.2 mg/mL) and sample B (16.73±0.02 mg/mL) (Figure 3a, b). ACE-I activities and IC_{50} values of kefir samples were determined on days 1, 7, 14, 21 and 28 of storage and the results were shown in Figure 3(a, b). The results were expressed as % inhibition and IC_{50}. With the progress of the storage period, it was found that the kefir had an increase in ACE-I activities and this increase was statistically significant ($P<0.01$). Sample B (including *L. helveticus*) showed strongest inhibition activity (57.28%±1.03) and IC_{50} value of 19.86±0.1 mg/L on the first day. The use of *Lactobacillus* bacteria in kefir production has been associated with a significant increase in ACE-I activity and it has been determined that the bacterium that maximizes ACE-I activity was *L. plantarum.* The highest ACE-I activity revealed that sample C on the 28 day and ACE-I activities of other samples was 76.47%, 84.95%, 87.33% and 85.57% respectively. IC_{50} values between 30.27±1.13 and 19.71±0.73 mg/L. *L. plantarum* showed the strongest ACE-I activity of 87.33±0.67% (IC_{50}=19.71±0.73 mg/L) on the last storage day. We may explain that higher ACE-I activity associated with an extensive proteolysis activity which may an increase in the intensity of peptides recognized as potential ACE inhibitors due to the adjunct culture addition especially lactobacilli. This limitation occurs once ACE-I activity depends on antihypertensive peptides' presence, which in turn relies on the balance between the release of bioactive peptides and the cleavage of these fractions in amino acids and inactive peptides (Ahiara et al. 2015; Rutella et al. 2016). Fermentation by lactic acid bacteria could positively contribute to ACE-I activity, as revealed in this study, and as reported by Aihara et al. (2005) who described an increase in activity following fermentation of powdered milk with *L. helveticus.* ACE-I activity showed a significant increase in milk fermented by *L. plantarum* K25 (Zhang et al. 2020). The addition of *Lactobacillus* may technological strategy that can be used in kefir processing to favor the bioactive potential of this product. Almost all the bioactive peptide sequences reported in many studies, including those displaying ACE-I activity, are derived from dairy products, especially fermented milk products, and classified as antihypertensive peptides.

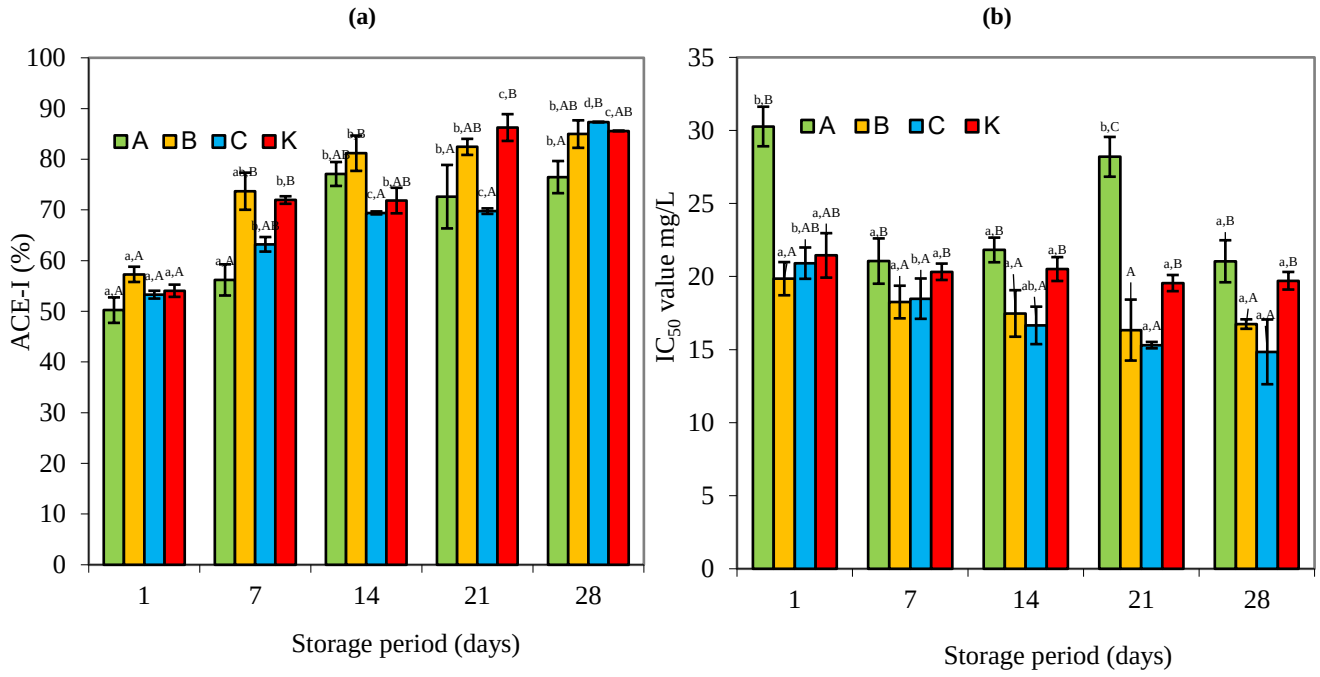


Figure 3- (a) ACE-I activity of Samples. sample K, kefir granule containing; sample A: kefir granule+ *L. bulgaricus*; sample B: kefir granule + *L. helveticus*; sample C: kefir granule + *L. plantarum*. 3 (b) IC_{50} value (mg/L Bovine Serum Albumin) for ACE-I activity of kefir samples. The capital letters A and B indicate means that significantly differ at P<0.01 between samples kefir. The lower case letters a and b indicate means that significantly differ at P<0.01 between days

3.4. Determination of antimicrobial activity and characterization of antimicrobial peptide by LC/MS-MS

Kefir contains several metabolites and inhibitors such as organic acid, peroxide hydrogen, ethyl alcohol, diacetyl, peptide, and bacteriocins. Antimicrobial activity is derived from lactic acid and other metabolites (Teneva et al. 2017). The metabolites are interacting to improve antimicrobial activity during kefir fermentation. The antimicrobial properties of kefir can be its metabolites produced by kefir microorganisms, such as exclusive peptides and exopolysaccharides (Kim et al. 2016). The indicator and pathogenic microorganisms were selected to determine the antimicrobial activity and disk diffusion test was used to certain the antimicrobial activity of the sample extracts (A, B and C) and lyophilized WSN fractions (<3 kDa). The antimicrobial effect against some microorganisms as inhibition zone diameter (mm) of kefir extract and peptide fraction was given in Table 2. Kefir extract A, B and C showed broader spectrum antimicrobial activity, inhibiting *S. aureus, S. warneri, S. xylosus, B. cereus, E. coli, E. fecalis, Enterobacter.* Sample K (control), on the other hand, showed a narrower spectrum antimicrobial activity by inhibiting *S. aureus, E. coli* and *E. fecalis.* However, in the sample K, it set forth the finding that the proteolysis during the kefir fermentation is not high enough to form antimicrobial peptides. Also, the microbial diversity can be different in each fermentation process of kefir. *Lactobacillus* strains that are generally consumed as probiotics and these bacteria may possess antimicrobial activity. This study has shown that the peptide fraction (<3k Dda) from kefir fermented using *Lactobacillus* adjunct culture presented high inhibition of the growth of *S. aureus, S. warneri* and *S. hominis* from 28th day of storage. The antimicrobial effect wasn't observed against *C. albicans* for all the kefir extract and peptide fractions in this study. Taşkın & Akköprü (2020) was reported that the antimicrobial activity of kefir may be due to the antimicrobial substances present in the supernatants. It was reported that kefir possessed an antibacterial activity against *E. coli* D157: H7 and *S. aureus* (Kivanc & Yapici 2018). In a study similar to, it was reported that kefir inhibited *B. subtilis, S. aureus* and *E. faecalis* and *S. enteritidis* but did not inhibit *C. albicans* (Chifiruc et al. 2011). The higher antibacterial activity of kefir supplemented with lactobacilli may be attributed to the hydrolysis of proteins and its contribution to antimicrobial peptide release by the enzymes of lactobacilli. It was reported that *L. delbrueckii* subsp *bulgaricus* has the antimicrobial activity against some of pathogenic bacteria including *E. coli* and *Shigella*. Presence of lactic acid along with the organic acids, bioactive peptides and bacteriocins may affect the antimicrobial activity of *L. bulgaricus* (Zaeim et al. 2014). Antimicrobial activity of *L plantarum* against some patogenic bacteria has already been established. Antibacterial property of *L. plantarum* might be due to its ability production of antibacterial compounds, which may inhibit the growth of harmful and pathogenic bacteria (Monteiro et al. 2019). The microbial diversity is responsible for the biological activities depending on the amount and various of *Lactobacillus* in kefir, their microbial diversity is different, and therefore, its antibacterial activity also changes. It means that peptides produced by *Lactobacillus* are more in number than peptides produced by lactic acid bacteria. This result is strongly related to the type and amount of peptidases. To make use of casein in milk, lactic acid bacteria firstly hydrolyze casein into oligopeptides by cell wall proteases and then transport it into cells through a specific oligopeptide transport system, further degrading oligopeptides into smaller peptides and amino acids by intracellular peptidases to provide for the growth and utilization of bacteria. At the same time, intracellular peptides are not only from casein hydrolysis, but also from the *Lactobacillus* itself because of existing bacteria protein degradation (Savijoki et al. 2006). Additionally, to pharmaceutical drugs, without side effects bioactive peptides considered as alternatives daily with a variety of protein-rich foods can be consumed. Bioactive peptides as potent natural ingredients it will contribute to its use in foods. Firstly, separation and detection of antimicrobial peptides should be done in kefir

metabolomics. It is important to investigate this mechanism. Additionally, future studies will focus on sequences of peptides identified in these fractions.

Separation of antimicrobial peptides was analyzed by HPLC. HPLC is a widely used method for the purification of peptides (Ahn et al. 2014; Zheng et al. 2018). The F2 fraction was further separated using HPLC. The chromatographic profile was given in Figure 1b. Total eight fractions were obtained and numbered sequentially F1-8. The antimicrobial activity of the F2 fraction was determined and the results were given (Table 2). F2 fraction had strong antimicrobial activity and no antimicrobial activity was observed in other fractions. Therefore, an attempt was made to determine the peptide characterization of the F2 fraction. In this report, we examined the MS/MS spectra of the protein fractions. The peptide fraction named F2 was determined to have been an antimicrobial inhibitory activity against three bacteria (*S. hominis*, *S. warneri* and *S. aureus*) and was collected from RP-HPLC. The peptide characterization was obtained by direct LC-MS/MS measurement in the m/z range from 100 to 1900 Da. The tuning of peptides was performed through continuous infusion of standards into ESI (positive electrospray ionization) mode source in positive mode at a flow rate of 10 µL/min. We reported the product and precursor ions by determining the m/z value of the peptide fractions that had an antimicrobial effect on *S. aureus*, *S. warneri* and *S. hominis.* Total ion spectra by LC-MS/MS of fraction 2 with three major peaks was given in Figure 4a and 4b for samples A, B and C. Considering the comparison with Figure 4a ve Figure 5, we can see that the peaks of A and C are larger in amount than B and K. The Q3 mass was predominant among the full scan of protonated precursors and the product ions of precursor ions were monitored further. The product ion scan yielded the following predominant fragment ions at m/z values 339.381 and 308 for precursor ions for samples A, B and C (Figure 5).

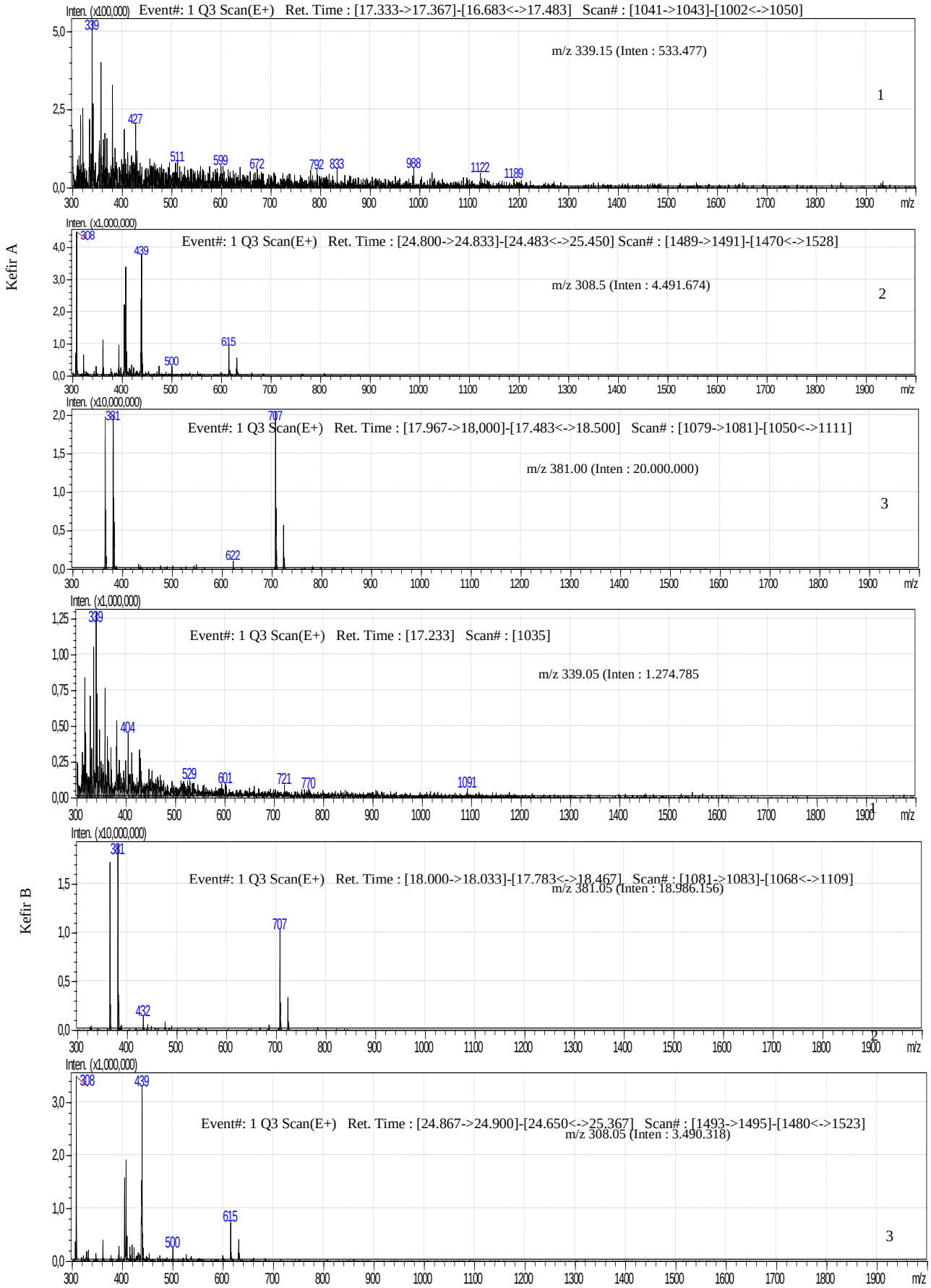
Bioactivity of protein fractions is related to amino acid composition, sequence, size and configuration of peptides (Rutella et al. 2016). Future studies will focus on sequences of peptides identified in this fraction.

Table 2 (a)-Antibacterial activity of peptide fractions (F2) in term of inhibition zone (IZ) in mm (b)Antibacterial activity of sample extract in term of inhibition zone (IZ) in mm

Bacteria	***A***		***B***		***C***		***K***		***Oxytetracycline***	
	(a)	***(b)***	***(a)***	***(b)***	***(a)***	***(b)***	***(a)***	***(b)***	***(a)***	***(b)***
S. epidermidis	-	-	-	-	-	-	-	-	+++	+++
S. aureus	+	++	+	++	+	++	-	+	++	++
S. warneri 95052	-	+	+	++	-	+	-	-	+	+
S. xylosus	-	++	-	+++	-	++	-	-	+++	+++
S. flexneri RSKK 184	-	-	-	-	-	-	-	-	+	+
B. cereus RS 863	-	+	-	+	-	-	-	-	+++	+++
E. coli	-	++	-	++	-	++	-	+	++	++
E. faecalis	-	++	-	++	-	++	-	++	++	++
C. albicans ATCC 04055	-	-	-	-	-	-	-	-	ND	ND
Str. mutans	-	-	-	-	-	-	-	-	+++	+++
Enterobacter	-	+	-	+	-	-	-	-	+++	+++
S. enteritidis	-	-	-	+	-	-	-	-	+++	+++
S. hominis	-	-	-	-	++	-	-	-	+++	+++

Sample K, kefir granule containing; sample A: kefir granule + *L. bulgaricus*; sample B: kefir granule + *L. helveticus*; sample C: kefir granule+ *L. plantarum.* (+) inhibition positive; (+) inhibition diameter 4-10 mm; (++) inhibition diameter 11-19 mm; (+++) inhibition diameter greater than 20 mm; (-) Inhibition negative.

(4a)


Kefir A
Kefir B
Inten. (x100,000) Event#: 1 Q3 Scan(E+) Ret. Time : [17.333->17.367]-[16.683<->17.483] Scan# : [1041->1043]-[1002<->1050]
m/z 339.15 (Inten : 533.477)
1
Inten. (x1,000,000) Event#: 1 Q3 Scan(E+) Ret. Time : [24.800->24.833]-[24.483<->25.450] Scan# : [1489->1491]-[1470<->1528]
m/z 308.5 (Inten : 4.491.674)
2
Inten. (x10,000,000) Event#: 1 Q3 Scan(E+) Ret. Time : [17.967->18,000]-[17.483<->18.500] Scan# : [1079->1081]-[1050<->1111]
m/z 381.00 (Inten : 20.000.000)
3
Inten. (x1,000,000) Event#: 1 Q3 Scan(E+) Ret. Time : [17.233] Scan# : [1035]
m/z 339.05 (Inten : 1.274.785
1
Inten. (x10,000,000) Event#: 1 Q3 Scan(E+) Ret. Time : [18.000->18.033]-[17.783<->18.467] Scan# : [1081->1083]-[1068<->1109]
m/z 381.05 (Inten : 18.986.156)
2
Inten. (x1,000,000) Event#: 1 Q3 Scan(E+) Ret. Time : [24.867->24.900]-[24.650<->25.367] Scan# : [1493->1495]-[1480<->1523]
m/z 308.05 (Inten : 3.490.318)
3
m/z

(4b)

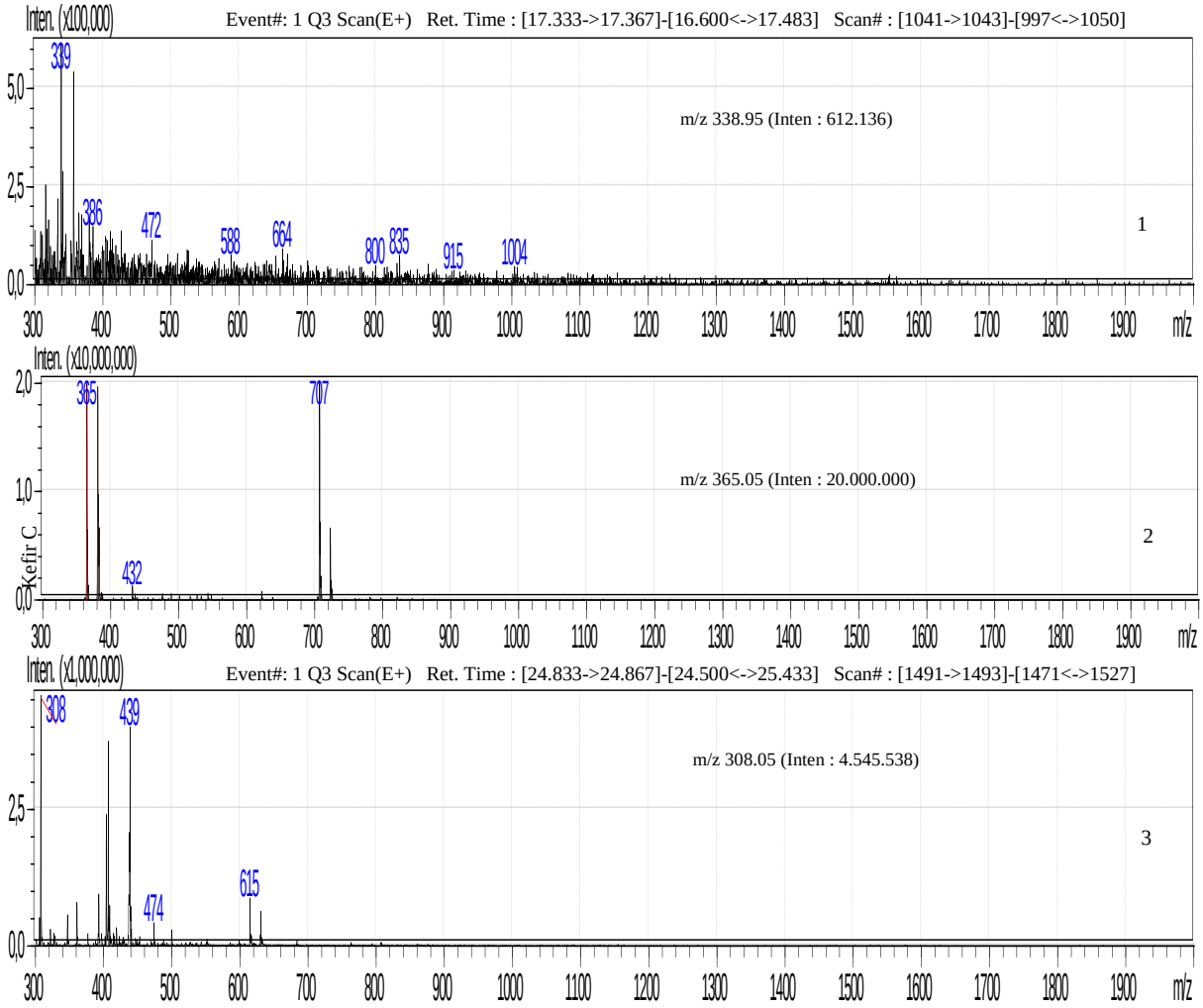


Figure 4- (a) Intensity chromatogram of LCMS/MS spectrum of ions from fraction 2 in sample A; Intensity chromatogram of LCMS/MS spectrum of ions from fraction 2 in sample B. (b) Intensity chromatogram of LCMS/MS spectrum of ions from fraction 2 in sample C.

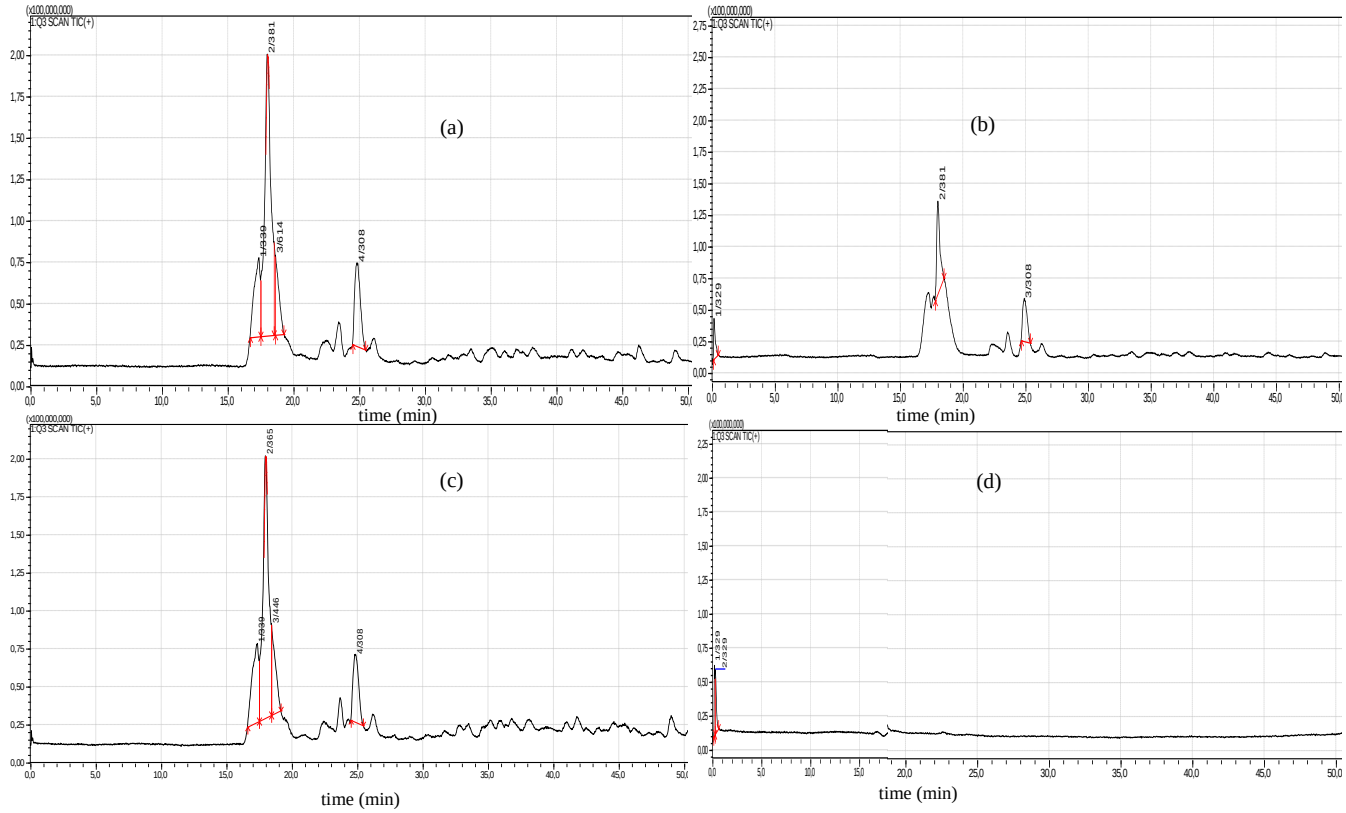


Figure 5- Illustrates total ion chromatogram of fraction 2 with three major peaks. The MS/MS spectrum of peak 1 at 339 m/z, peak 2 at 381 m/z in where the ion of 381 m/z was the most abundant for samples B and A, peak 3 at 308 m/z was shown. The MS/MS spectrum of peak 1 at 339 m/z, peak 2 at 365 m/z in where the ion of 381 m/z was the most abundant for C kefir samples, peak 3 at 308 m/z was shown (a) kefir granule + *L. bulgaricus*; (b) kefir granule + *L. helveticus*; (c) kefir granule + *L. plantarum,* (d) kefir granule containing

4. Conclusions

As a result, the use of adjunct culture in addition to kefir grains obtained better results than the traditional kefir method (produced with kefir grains alone) in terms of proteolysis and bioactive properties. The results show that the ACE-I activity was higher in kefir samples that produced *Lactobacillus* adjunct cultures. It was determined that the peptide fractions (for fraction F2) of the kefir samples produced by the addition of *Lactobacillus* have an antimicrobial effect compared with peptide fractions of sample K. In addition, characterization of the peptide fraction named F2, which has antimicrobial activity against *S. hominis*, *S. warneri* and *S. aureus*, was achieved by LC-MS/MS. These findings supported that the kefir which probiotic fermented product has shown antihypertensive potential as a functional food and indicated application of the peptides as bio-preservative. The shelf life of food products can be extended by using these antimicrobial metabolites produced by selected microorganisms.

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