

Identification of bacterial microbiota of traditional homemade vinegars

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Summary

In this study, microbiological analyses were performed on 41 samples of homemade vinegar to obtain information on the microbiota that might play a role in its fermentation. For this aim total aerobic mesophilic bacteria, yeasts and moulds, enterococci, coliform bacteria, probable acetic acid bacteria counts, pH and titratable acidity were investigated. Ninety-eight bacterial isolates were obtained from glucose-yeast-extract-calcium carbonate (GYC) medium. The isolates were classified using repetitive-element polymerase chain reaction (rep-PCR) technique with GTG5 primer. Identification of the isolates was performed by partial 16S rDNA sequencing. *Bacillus*, *Brevibacillus parabrevis*, *Acetobacter okinawensis*, *Paenibacillus*, *Lactobacillus casei*, *Acetobacter pasteurianus* and *Acinetobacter johnsonii* were found in the microbiota of vinegar samples. Low acidity of the product and the composition of the microbiota suggested that the production of vinegar at home was often not successful and the product obtained was actually not vinegar.

Keywords

homemade vinegar; acetic acid bacteria; identification; 16S rDNA

According to the definition of FAO-WHO [1], vinegar is a liquid suitable for human consumption obtained from agricultural raw materials (grapes, fruits or berries, cider) or containing sugar and/or starch by using a two-stage fermentation process, namely alcoholic fermentation and acetic acid fermentation. The first stage of vinegar production is alcoholic fermentation. It is mainly carried out by *Saccharomyces cerevisiae* strains under anaerobic conditions. The yeasts produce ethanol, CO₂ and small amounts of glycerol, acetic acid and some higher alcohols. The second stage, acetic acid fermentation, is mainly carried out *Acetobacter*, *Gluconobacter* and *Gluconoacetobacter* species under aerobic conditions. These bacteria produce a variety of organic acids, including acetic, tartaric, lactic, malic and citric acids. Acetic acid occurs at the highest rate among these acids [2–4].

Vinegar is a common food product. It can be produced from various types of raw materials in almost every country [5]. A flow chart of the production of traditional homemade vinegar is given in Fig. 1. In ancient times, vinegar was considered an expensive and luxurious drink in countries where fermented vinegar production could not be

widespread. However, today it is used in salads, in the preparation of various sauces, mayonnaise, ketchups and mustard, and even as a medicine in the treatment of some diseases. At the same time, vinegar accelerates the process of softening and relaxation of meat or softens fish bones. It is used in the preparation of fish and various seafood as well as pickles in the fruits and vegetables industry. In addition, it improves the sensory properties and preserves the food by giving it acidity [6, 7]. On the other hand, vinegar has beneficial effects on health when consumed regularly, which is due to its antimicrobial, antidiabetic, antioxidative, antiobesity and antihypertensive activities. These effects are due to the presence of various pharmaceutically active components as polyphenols, micronutrients and other bioactive compounds [8, 9]. Raw materials, water, nutrients added for the growth of bacteria, vinegar production method and maturation time affect the composition of the vinegar. The pH value of the vinegar varies between 2.0 and 3.5 depending on the fermentation time and conditions. Vinegar contains acetic acid, which plays a major role in the formation of the sharp and sour taste specific to vinegar. The

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side-products in vinegar are organic acids, mineral substances, proteins and carbohydrates, together with volatiles such as esters, aldehydes, methylglyoxal, dihydroxyacetone, 2-buteneglycol, acetyl-methylcarbinol, or diacetyl [2, 10, 11]. Aromatic compounds have a significant effect on vinegar quality. The use of a mixed culture and the prolonged contact of the vinegar with wood during ripening shape the vinegar aroma [12].

In this study, 41 samples of homemade vinegars produced using traditional methods were examined to determine the presence of vinegar bacteria involved in fermentation. Strains playing a role in the formation of acid in the mixed microflora of the samples were isolated and characterized by repetitive-element polymerase chain reaction (rep-PCR). Identification of the isolates was achieved by 16S rDNA sequencing.

MATERIALS AND METHODS

Vinegar samples

In the study, 41 homemade vinegar samples produced from diverse raw materials were obtained from various regions of Turkey (Türkiye; Fig. 2, Tab. 1). The samples were brought to the microbiology laboratory of the Department of Food Engineering, Ataturk University Erzurum, Turkey (Türkiye) and kept at 4 ± 1 °C under refrigerator conditions until analysed. on the same day.

Microbiological analysis

To determine aerobic plate counts, 0.1 ml of appropriate dilutions were spread on plates of plate count agar (PCA; Merck, Darmstadt, Germany) and the plates were incubated at 30 °C for

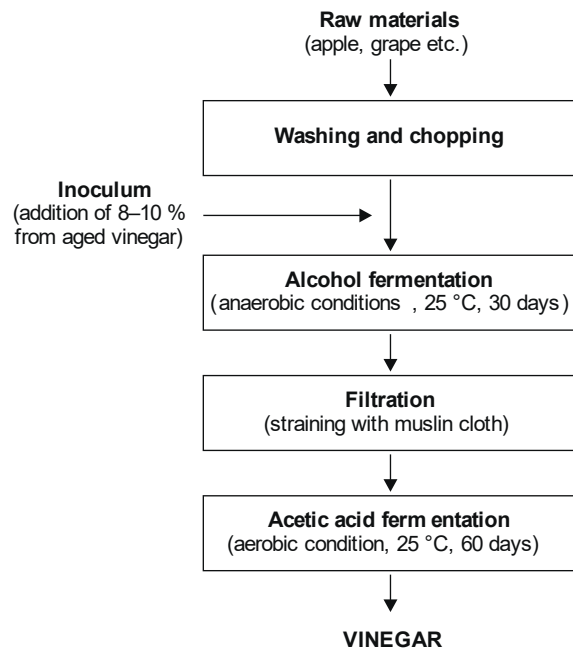


Fig. 1. General flow chart of traditional homemade vinegar production [31–33].

72 h [13]. Violet red bile agar (VRB, Merck) was used to determine counts of coliform bacteria, with incubation at 37 °C for 24 h counting typical colonies [14]. To determine yeasts and moulds counts, potato dextrose agar (PDA, Merck) was sterilized and acidified with 100 g·l⁻¹ sterile tartaric acid solution. Incubation took place at room temperature (± 25 °C) for 5 days [4, 15]. Kanamycin aesculin azide agar (KAA, Merck) was used to determine counts of *Enterococcus* genus, with incubation at 35–37 °C 24 h. Enumeration was carried



Fig. 2. Cities in Turkey (Türkiye) from where the vinegar samples were supplied.

Tab. 1. General information about the homemade vinegar samples.

Sample No.	Raw material	Region	Age of samples (months)
1	Apple cider	Adana	12
2	Apple cider	Adıyaman	1
3	Grape	Aydın	11
4	Turnip	Adana	10
5	Apple cider	Malatya	10
6	Apple cider	Adana	15
7	Grape	Aydın	18
8	Grape	Aydın	18
9	Grape	Antalya	12
10	Apple cider	Erzurum	1
11	Grape	Antalya	8
12	Grape molasses	Antalya	8
13	Grape	Tokat	4
14	Apple/grenade mix	Erzurum	3
15	Red cabbage	Erzurum	3
16	Grape	Antalya	12
17	Honey	Erzurum	3
18	Fig	Ankara	6
19	Grape	Ankara	6
20	Apricot	Ankara	7
21	Lemon	Ankara	2
22	Rose hip	Erzurum	18
23	Grape	Rize	18
24	Apple cider	Antalya	7
25	Apple cider	Rize	6
26	Persimmon	Niğde	3
27	Artichoke	Niğde	3
28	Hawthorn	Niğde	3
29	Grape	Rize	4
30	Apple cider	İstanbul	3
31	Apple cider	İstanbul	24
32	Mulberry	İstanbul	48
33	Mulberry	İstanbul	24
34	Apple cider	Erzurum	2
35	Grape	Ankara	4
36	Apple cider	Ankara	6
37	Apple cider	Erzurum	8
38	Apple cider	Ankara	8
39	Grape	Erzurum	4
40	Pear	Rize	2
41	Apple cider	Ankara	9

out considering the black and halo colonies observed at the end of the incubation [16].

In order to choose the most suitable medium for counting and isolation of probable acetic acid bacteria, microbiological cultures were made from the same vinegar samples on three different media. These were glucose, yeast extract and calcium carbonate (GYC) agar, mannitol, yeast extract and peptone (MYP) agar and modified acetobacter medium (MAB) agar by providing the same environmental conditions. MAB was prepared by us according to the literature. In addition, cycloheximide ($100 \text{ mg}\cdot\text{l}^{-1}$) was added to the medium to prevent the growth of yeasts and moulds. It was added as a homogenous mixture after sterilizing the medium. Inoculation was carried out using appropriate dilutions and plates were incubated at 30°C for 48 h. Counting and isolation were performed considering yellow and green colonies and its microscobic characteristics [16, 17]. Out of the three media tested, GYC was found to be the most suitable medium regarding microbial counts and diversity. The composition of the media is presented in Tab. 2.

Molecular analysis of isolates

Repetitive-element polymerase chain reaction (rep-PCR) is used extensively to reveal the diversity of fermented foods with generated groups being identified by sequencing [18]. DNA isolation from acetic acid bacteria isolates was performed from young cultures. In order to determine the differences on species and strain levels, the obtained DNA samples were subjected to GTG5 fingerprinting [19]. One representative strain from each of the groups was selected and evaluated by sequencing to identify species and strain differences.

In order to perform DNA isolation, bacterial suspensions were washed twice with phosphate buffer ($0.01 \text{ mol}\cdot\text{l}^{-1}$ phosphate buffer, $0.0027 \text{ mol}\cdot\text{l}^{-1}$ potassium chloride and $0.137 \text{ mol}\cdot\text{l}^{-1}$ sodium chloride, pH 7.4 at 25°C) and once with ultrapure water (Lonza, Verviers, Belgium). After centrifugation at $5000 \times g$ for 3 min. 0.5 ml of $6 \text{ mol}\cdot\text{l}^{-1}$ urea and 0.1 ml of $100 \text{ g}\cdot\text{l}^{-1}$ sodium dodecyl sulfate (SDS; Sigma, Steinheim, Germany) were added to the cell suspension and incubated at 37°C for 20 min. After the samples were kept at boiling temperature for 5 min, they were centrifuged at $8000 \times g$ at 25°C for 10 min. The pellet was incubated in the presence of 0.1 ml $0.2 \text{ mol}\cdot\text{l}^{-1}$ NaOH at 37°C for 10 min. In order to separate non-nucleic acid cellular parts, centrifugation was carried out at $3000 \times g$ at 25°C for 3 min. For DNA purification, 2.5 volumes of absolute alcohol ($\geq 99.9\%$, Merck) was added and the

samples were kept at -20°C for 2 h. Samples taken out of the freezer were centrifuged at $13\,000 \times g$ at 4°C for 15 min and washed with 70% ethanol. The pellet was dried, DNA was resuspended in $20\ \mu\text{l}$ Tris-EDTA (TE, pH 8.0; Sigma) and stored at 4°C [19]. The quality and concentration of DNA solutions were determined spectrophotometrically at 260 nm and 280 nm (Epoch Microplate Spectrophotometer, BioTek Instruments, Winooski, Vermont, USA) [20].

The PCR mixture contained GTG5 primer ($1\ \text{mmol}\cdot\text{l}^{-1}$; Oligomer, Ankara, Turkey (Türkiye)), $2\times$ EcoTaq Master Mix ($12.5\ \mu\text{l}$; EcoTech, Erzurum, Turkey (Türkiye)), template DNA solution (final concentration $2.5\ \text{ng}\cdot\mu\text{l}^{-1}$) and deionized water ($9\ \mu\text{l}$), the total volume was adjusted to $25\ \mu\text{l}$. The prepared samples were subjected to PCR with initial denaturation at 98°C for 30 s; followed by 30 cycles of denaturation at 94°C for 10 s, annealing at 59°C for 15 s, polymerization at 72°C for 15 s and final polymerization at 72°C for 1 min. PCR products were analysed by electrophoresis in a $15\ \text{g}\cdot\text{l}^{-1}$ agarose gel (Sigma) and visualized by staining with ethidium bromide. Size of DNA fragments was estimated using Plus DNA Ladder (Solis BioDyne, Tartu, Estonia) [18].

Strains with similar GTG5 profiles were grouped and one representative was selected for each group. From those strains, PCR targeted to 16S rDNA was carried out with primers 8F ($5'\text{-AGA GTT TGA TCC TGG CTC AG-3}'$) and 1541R ($5'\text{-AAG GAG GTG ATC CAG CCG CA-3}'$) in similar conditions as stated above, just with annealing at 65°C for 15 s. The PCR products were subjected to DNA sequencing (ABI 3130 XL Genetic analyser, Applied Biosystems, Bedford, Massachusetts, USA). The results were analysed using Basic Local Alignment Search Tool (BLAST) algorithm and the GenBank database (National Center for Biotechnology Information, Rockville Pike, Bethesda, Maryland, USA).

pH and titratable acidity

The pH values of the vinegar samples were determined by immersing the probe (HI 221, Hanna, Nussfalau, Romania) into homogenized samples.

Total acidity was determined by titration using a standardized solution ($0.1\ \text{mol}\cdot\text{l}^{-1}$ NaOH) and phenolphthalein as indicator [21].

Statistical analysis

Statistical analyses were carried out using SPSS version 20.0 package program (SPSS, Chicago, Illinois, USA). Descriptive statistics, multivariate and correlation tests were used to determine the differences between the results and means. Analy-

Tab. 2. Composition of the media used in the preliminary experiment for isolation of probable acetic acid bacteria.

Medium	Component	Concentration [$\text{g}\cdot\text{l}^{-1}$]
GYC	D-Glucose	50
	Yeast extract	10
	CaCO_3	15
	Bromocresol purple	0.05
	Agar	15
MYP	D-Mannitol	25
	Yeast extract	5
	Peptone	3
	Agar	15
MAB	D-Glucose	20
	Yeast extract	5
	Ethyl alcohol	20
	$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	0.25
	KH_2PO_4	1
	$(\text{NH}_4)_2\text{SO}_4$	3.3
	Agar	15

GYC – glucose, yeast extract and calcium carbonate agar, MYP – mannitol, yeast extract and peptone agar, MAB – modified acetobacter medium agar.

ses in the study were performed in triplicate and significant differences were identified at $p < 0.05$.

RESULTS AND DISCUSSION

Microbiological parameters

Results of microbiological analyses of 41 traditional vinegar samples are given in Tab. 3. When the results were examined, it was determined that the highest aerobic plate count value of the vinegar samples was $7.82\ \log\ \text{CFU}\cdot\text{ml}^{-1}$ and the lowest value was $< 1\ \log\ \text{CFU}\cdot\text{ml}^{-1}$. The mean value was at the level of $3.53\ \log\ \text{CFU}\cdot\text{ml}^{-1}$. It was seen that the counts of yeasts and mould ranged from $< 1\ \log\ \text{CFU}\cdot\text{ml}^{-1}$ to $7.99\ \log\ \text{CFU}\cdot\text{ml}^{-1}$, and the mean value was $2.87\ \log\ \text{CFU}\cdot\text{ml}^{-1}$. It

Tab. 3. Microbiological characteristics of vinegar samples.

	Min.	Max.	Mean
	[$\log\ \text{CFU}\cdot\text{ml}^{-1}$]		
Aerobic plate counts	< 1	7.82	3.53 ± 2.78
Yeasts and moulds	< 1	7.99	2.87 ± 2.59
Coliforms	< 1	2.40	0.17 ± 0.55
Enterococci	< 1	3.78	0.95 ± 1.50
Probable acetic acid bacteria	< 1	7.78	2.77 ± 2.56

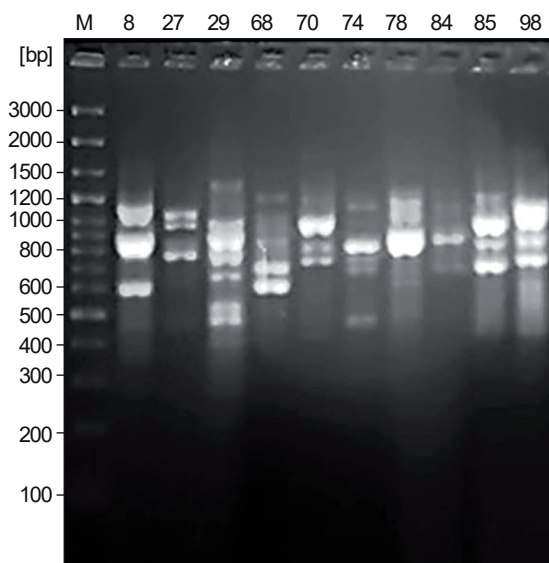


Fig. 3. Rep-PCR fingerprint profiles of the representative ten isolates from vinegar samples.

was determined that the coliform counts of the samples were between $< 1 \log \text{CFU} \cdot \text{ml}^{-1}$ and $2.40 \log \text{CFU} \cdot \text{ml}^{-1}$, and the mean value was $0.17 \log \text{CFU} \cdot \text{ml}^{-1}$. Coliforms were detected in four samples. This result suggested that vinegar is generally safe. It was determined that the *Enterococcus* counts in the samples ranged from $< 1 \log \text{CFU} \cdot \text{ml}^{-1}$ to $3.78 \log \text{CFU} \cdot \text{ml}^{-1}$, and the mean was

$0.95 \log \text{CFU} \cdot \text{ml}^{-1}$. Enterococci were observed in twelve vinegar samples, the highest counts were determined in sample No. 15. Probable acetic acid bacteria values were between $< 1 \log \text{CFU} \cdot \text{ml}^{-1}$ and $7.78 \log \text{CFU} \cdot \text{ml}^{-1}$ and the mean value was $2.77 \log \text{CFU} \cdot \text{ml}^{-1}$.

Molecular characterization of isolates

Altogether 98 isolates were obtained from the vinegar samples and rep-PCR analyses were performed with these isolates. As a result, 10 groups were formed and one representative sample was selected from each group (Fig. 3). 16S rDNA sequence analyses of the selected strains from each group were carried out (Tab. 4).

Bacterial composition of homemade vinegar is shown in Fig. 4. The dominant bacterium in the microbiota of vinegar samples was the genus *Bacillus* (40 %) and the second was *Brevibacillus parabrevis* (36 %). Although acetic acid bacteria play an active role in vinegar fermentation, *Acetobacter okinawensis* was the third most isolated species (17 %). *Lactobacillus casei* was found only in sample No. 37, *Paenibacillus* sp. was determined in sample No. 33 and *Acinetobacter johnsonii* strains were found in sample No. 33. These results are in agreement with those of different studies, where it was determined that vinegar samples contained *Bacillus*, *Paenibacillus* and *Lactobacillus* genera [2, 22, 23]. Various microorganisms from fruits and

Tab. 4. Identification results of GYC agar isolates from vinegar samples.

Group	Genetic identification result	Genetic similarity ratio [%]	Sequence ID number	Total number of representative strains	Representative strain codes
1	<i>Bacillus</i> sp.	100	JN819585.1	2	8*, 9
2	<i>Bacillus</i> sp.	99	HM566796.1	36	2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27*, 28, 32, 33, 34, 35, 38, 39, 43, 50, 51, 53, 54
3	<i>Acetobacter okinawensis</i>	99	AB665065.1	15	1, 29*, 30, 31, 36, 40, 41, 42, 45, 46, 47, 48, 49, 52, 55
4	<i>Brevibacillus parabrevis</i>	98	KX832701.1	35	56, 57, 58, 59, 60, 61, 62, 64, 65, 66, 67, 71, 72, 73, 74*, 76, 77, 79, 80, 81, 82, 83, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 99, 100
5	<i>Paenibacillus</i> sp.	99	KX025137.1	3	63, 68*, 75
6	<i>Paenibacillus</i> sp.	99	FJ666319.1	2	69, 78*
7	<i>Lactobacillus casei</i>	99	KF029502.1	2	97, 98*
8	<i>Acetobacter pasteurianus</i>	97	MF179553.1	1	70*
9	<i>Bacillus</i> sp.	99	KY243896.1	1	84*
10	<i>Acinetobacter johnsonii</i>	99	KY606925.1	1	85*

GYC – glucose, yeast extract and calcium carbonate agar.

* – strains used in identification, representing the group with the same GTG5-profile.

the environment are part of the vinegar microbiota. Within the scope of the study, while it was expected that the majority of the isolates detected were acetic acid bacteria, only 15 of 98 isolates were *Acetobacter okinawensis* and 1 was *Acetobacter pasteurianus*. The genus *Bacillus* may limit the growth of acetic acid bacteria, because fermentation conditions are not fully controlled in homemade products.

Since the fermentation conditions cannot be controlled completely, especially in homemade traditional products, various microorganisms from fruits and the environment are also part of the microbiota and perhaps limit the growth of acetic acid bacteria. The predominance of *Bacillus* spp., *Brevibacillus* spp. and *Paenibacillus* spp. strains in samples 8, 27, 28 with high acetic acid content suggests that these bacteria remained alive. In general, the isolation of such spore-forming bacteria from this type of samples is taken as related to their resistance to environmental and vinegar conditions during all production stages [24, 25]

LOGAN and DE VOS [26] reported that *Brevibacillus* and *Paenibacillus* genera were in the same family (Paenibacillaceae). In addition, it is also mentioned that *Paenibacillus* and *Brevibacillus* genera are soil-borne and endospore-forming bacteria [8, 26]. Therefore, the presence of these two genera in the vinegar samples strengthens the opinion that they contaminate the vinegar with the fruit materials used in vinegar production and their vitality continues after acetic acid fermentation [27].

In previous studies of vinegar samples, *Acetobacter* spp., *Gluconobacter* spp., *Gluconoacetobacter* spp. and *Komagataeibacter* spp. were more commonly isolated [28, 29]. On the other hand, although *Bacillus* spp. and *Lactobacillus* spp. strains were isolated from some vinegars, these were not the dominant species. In a traditional Chinese vinegar, it was reported that *Bacillus* spp. and *Lactobacillus* spp. were the dominant contaminants, causing turbidity as a marker of spoilage [30].

On the basis of these results, the homemade vinegar samples were not acceptable in terms of vinegar quality criteria. This seems to be due to the dominance of non-acetic acid bacteria instead of acetic acid bacteria in vinegar fermentation.

pH and titratable acidity

AKTAN and YILDIRIM [2], reported that the pH value of the vinegar varies between 2.0 and 3.5. In our study, the pH values of the vinegar samples were generally at the expected level, except for sample No. 33, which was one of the two mulberry

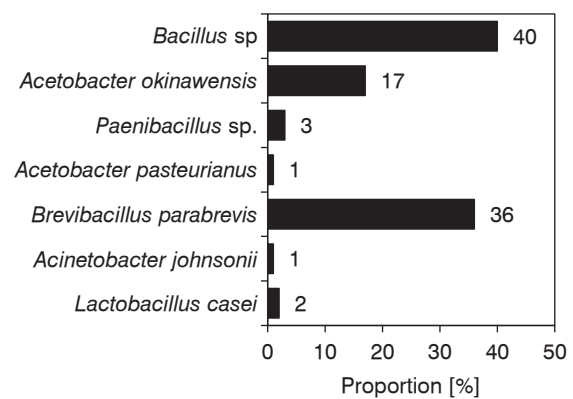


Fig. 4. Bacterial diversity of vinegar samples.

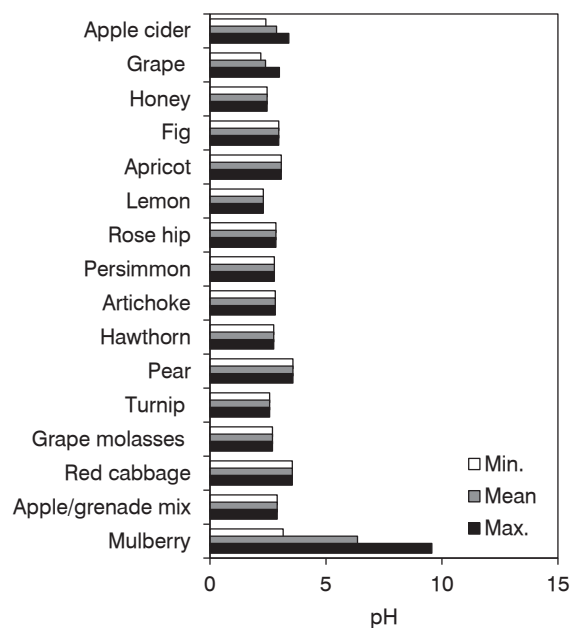


Fig. 5. pH values of the vinegar samples.

samples (Fig. 5). The minimum, maximum and mean pH values of all the samples were detected as 2.19, 9.57 and 2.96, respectively.

FAO/WHO requires total acids of vinegar to be 50–60 g·l⁻¹ [1]. The acidity of the vinegar samples in our study is shown in Fig. 6. It was observed that the acidity levels were generally low. The low acidity level strengthens the opinion that acetic acid fermentation in the products was very low or even did not occur. The fact that the low acidity level and the isolates obtained from vinegar samples contained various genera of bacteria suggest that there were problems in the traditional vinegar production. Only in a few samples (No. 7, 9, 12, 13, 16), the total acidity level was high. It is thought that this may be due to the high amount

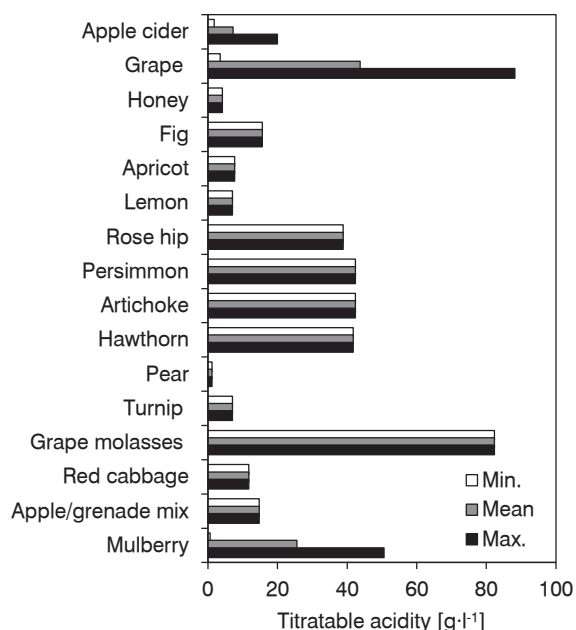


Fig. 6. Titratable acidity values of the vinegar samples.

of inoculant vinegar added to accelerate fermentation in those cases.

Gram-positive bacteria were also included in the isolation attempts and important information was obtained at revealing the microbiota of the vinegars. The first of these was that it is wrong to evaluate directly coloured colonies (yellow and green) as acetic acid bacteria in media such as GYC, where identification is made only based on the colour change. The second is the fact that various microorganisms from the fruits and the environment are also a part of the microbiota and perhaps limit the growth of acetic acid bacteria, since fermentation conditions cannot be fully controlled, especially in traditional homemade products.

CONCLUSIONS

When consumed regularly, vinegar exhibits health-promoting effects based on its antimicrobial, antidiabetic, antioxidant, antiobesity and anti-hypertensive properties. For this reason, interest in vinegar is increasing. In order to respond to this increasing interest, proper vinegar production needs to be ensured and more studies need to be carried out on vinegar production. In this study, the fermentation quality of homemade vinegar samples was attempted to be revealed especially by microbiological characterization. However, the differences between the minimum and maximum

values of microbiological counts, pH and acidity results showed that homemade vinegars are not produced according to the appropriate acetic acid fermentation method. The extensive isolation of genera such as *Bacillus*, *Brevibacillus* and *Paenibacillus* in homemade vinegars as well as the low mean and high standard deviation values observed in microbiological data supported this conclusion.

The formation of vinegar takes place as a result of a two-stage process, namely, alcoholic and acetic acid fermentation. People who traditionally make homemade vinegar should take this into account. After alcohol fermentation has taken place in anaerobic conditions, the alcoholic liquid must be kept in aerobic conditions. When compared to other fermented products, it is seen that the production of vinegar is not easy under home conditions due to difficulties such as the need for two different stages for the formation of vinegar, optimization of fermentation conditions and selection of suitable raw materials.

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