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Molecular Identification and Technological Properties of Acetic Acid Bacteria Isolated from Malatya Apricot and Home-Made Fruit Vinegars

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Acetic acid bacteria (AAB) are versatile organisms involved in the production of variety of fermented foods, such as vinegar and kombucha, and products of biotechnological relevance, such as bacterial cellulose. In the present study, Malatya apricot, a variety with protected designation of origin (PDO), and vinegar samples produced using various fruits were used to isolate AAB. The 19 AAB isolates obtained were typed using (GTG)5 fingerprinting, and the ones selected were identified by sequencing either 16S rDNA alone or in combination with 16S-23S rRNA internal transcribed spacer region or *ligA* gene. While all apricot isolates (n = 10) were *Gluconobacter cerinus*, vinegar isolates (n = 9) were composed of *Komagataeibacter saccharivorans*, *Acetobacter syzygii*, and possible two new species of AAB, *Komagataeibacter* sp., and *Gluconobacter* sp. (GTG)5 fingerprinting showed the presence of several genotypes of *G. cerinus* in the apricot samples. Screening for some technologically relevant properties, including thermotolerance, ethanol tolerance, and cellulose production capability, showed that all *Komagataeibacter* and some *Gluconobacter* isolates could tolerate the temperature of 35℃, and that vinegar isolates could tolerate up to 8% ethanol. One isolate, *Komagataeibacter* sp. GUS3 produced bacterial cellulose (1 g/l) and has the potential to be used for cellulose production.

Keywords: **Acetic acid bacteria (AAB), Malatya apricot, bacterial cellulose, *Komagataeibacter*, *Gluconobacter cerinus***

Introduction

Acetic acid bacteria (AAB) are aerobic Gram-negative microorganisms that taxonomically belong to the family *Acetobacteriaceae* of the class *Alphaproteobacteria* [1, 2]. They are commonly present on the surface of plants, fruits, and flowers, where they oxidize sugars and/or ethanol to produce organic acids [3]. AAB are the main microorganisms responsible for the production of fermented products, such as vinegar and kombucha [3]. Moreover, they are involved in the production of various biotechnological products, such as bacterial cellulose, which has potential food, pharmaceutical, and engineering applications [1, 4].

According to 2019 statistics, Turkey is the top apricot producer with ~847,000 T covering ~21% of worldwide production (FAO). The greatest Turkish apricot production is in the Malatya Province (391.801 T) [5]. The Malatya apricot (*Prunus armeniaca* L.) was registered as a protected designation of origin (PDO) in 2017. High total solid and soluble solid contents of the Malatya apricot are distinct from those grown in other regions of Turkey and in different countries [6]. The major sugars of the Malatya apricot variety is sucrose, the predomi-

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nant sugar, and glucose and fructose; sorbitol was also detected in considerable amounts [6]. To the best of our knowledge, the microbiota of this fruit has not been studied. Considering the high solid content and sugary nature, this fruit could represent a valuable source of microorganisms, including AAB.

In the present study, AAB were isolated from the Malatya apricot (PDO) and homemade vinegars produced using different fruits, namely, apple, red grape, white grape, orange, and wild pear. The isolates were identified molecularly by sequencing the 16S rRNA alone or in combination with either the 16S–23S rRNA internal transcribed spacer (ITS) region or *ligA*. In addition, industrially important traits such as thermotolerance, ethanol tolerance, and cellulose production ability were determined.

Materials and Methods

Samples

For AAB isolation, two Malatya apricot varieties-Hacihaliloglu and Hasanbey-were obtained from Malatya (Turkey) during the 2019 apricot season (June–July) and used in the present study. In addition to the apricots, five samples of homemade vinegar made using apple, red grape, white grape, orange, and wild pear were also used as samples for AAB isolation.

Isolation of AAB

Apricot samples (100 g) were homogenized using a stomacher (Bagmixer 400, Interscience, France) and allowed to ferment with their natural microbiota in a sterile jar at 30℃ for 15 d for selective growth of AAB under an alcohol-rich and a low pH environment [7, 8]. Serial dilutions of the fermented apricot homogenates and vinegar samples were prepared in peptone water (0.1% [w/v], Merck KGaA, Germany) and were plated on acetic acid medium (AAM) agar (1% glucose [Sigma-Aldrich, USA], 0.5% ethanol [Isolab Laborgeräte GmbH, Germany], 0.3% acetic acid [Sigma-Aldrich], 1.5% peptone [Merck], 0.8% yeast extract [Biolife, Italy], and 1.5% agar [Merck], all w/v except acetic acid and ethanol, which were v/v) [9, 10] and glucose yeast extract calcium carbonate (GYC) agar (10% glucose [Sigma-Aldrich], 1.0% yeast extract [Biolife], 2.0% calcium carbonate [Sigma-Aldrich], and 1.5% agar [Merck], w/v) [11]. Both media were supplemented with 0.4% (w/v) cycloheximide (Sigma-Aldrich) to inhibit yeast growth [9]. The petri plates were incubated at 30℃ for 72 h.

Morphologically different colonies were selected from the plates with 25–250 colonies and purified by streaking onto other plates with the corresponding isolation medium. Isolates were grown in isolation medium broths containing 20% glycerol and kept at -80℃ for long-term storage.

DNA extraction and polymerase chain reaction

DNA was isolated using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, USA, K182002) according to manufacturer's instructions for Gram-negative bacteria. The concentration and purity of the DNA samples were measured using the BioSpec Nano Spectrophotometer (Shimadzu, Japan).

The isolates were grouped by (GTG)5 fingerprinting analysis [12]. The polymerase chain reaction (PCR) mix contained 1X buffer, 0.2 mM deoxynucleoside triphosphate mix (Thermo Fisher Scientific), 4.0 μl 10 mM (GTG)5 primer, ~50 ng DNA, 2.5 U Dream Taq DNA polymerase (Thermo Fisher Scientific) and water up to 50 μl. PCR conditions were previously described [12]. PCR was conducted using a T100 thermal cycler (Bio-Rad Laboratories, USA). PCR products were separated on a 1.5% (w/v) agarose gel in 1X Tris base, acetic acid, and ethylenediaminetetraacetic acid (TAE) buffer at 30 V for ~5 h using a Wide Mini-Sub Cell GT electrophoresis system (Bio-Rad) and visualized using the Gel Doc EZ Imager (Bio-Rad).

One to several isolates from each (GTG)5 group were selected for molecular identification. The 16S rRNA gene was determined for these isolates using the primer sets 27F & 1492R [13]. 16S–23S rRNA gene intergenic transcribed spacer (ITS) region was also determined for *Komagataeibacter* and *Acetobacter* species using 16S its1 and 23S its2 primers [14], if they could not be discriminated well enough using 16S rRNA. For the isolates ELS3, ELS4, and ELS5, another marker-*dnaK*-was used with the primers dnaK500F3 and dnaK1710R5 [15], which led to the nonspecific amplification of *ligA*. The PCR mix was prepared the same as (GTG)5 fingerprinting PCR except that 2.0 μl forward and reverse primers (10 mM) were added. PCR conditions were as follows: initial denaturation at 94℃ for 7 min, 34 cycles of dena-

turation at 94℃ for 30 s, annealing at 50℃ for 30 s for 16S rRNA and 16S–23S rRNA ITS and 48℃ for 30 s for *ligA*, and extension at 72℃ for 1.5 min for 16S rRNA and *ligA* and at 72℃ for 1 min for 16S–23S rRNA ITS. The final extension was conducted at 72℃ for 10 min. PCR reactions were run on 0.8% agarose gels (w/v) in 1X TAE buffer at 80–95 V for ~45–60 min.

For sequencing, PCR products were purified using the GeneJet PCR purification kit (Thermo Fisher Scientific) according to manufacturer's instructions. Sequencing primers were the same as the PCR primers.

Sequences were deposited to GenBank as follows: 16S rRNA for *G. cerinus* HFG13, MZ396858, BFG1, MZ396859, BFA13, MZ396860, BFA9, MZ396861, BFA6, MZ396862, *Gluconobacter* sp. ELS5, MZ396865; 16S rRNA and 16S–23S rRNA ITS for *K. saccharivorans* GUS1, MZ396868 and MZ401138, *K. saccharivorans* GUS2, MZ396869 and MZ401139, *Komagataeibacter* sp. GUS3, MZ396870 and MZ401140, *A. syzygii* PS1, MZ396866 and MZ401136, *A. syzygii* AUS4, MZ396867 and MZ401137; 16S rRNA and *ligA* for *Gluconobacter* sp. ELS3, MZ396863 and MZ408910, *Gluconobacter* sp. ELS4, MZ396864 and MZ408911.

Fingerprinting analyses

(GTG)5 fingerprinting patterns of the isolates were analyzed using temporary BioNumerics (Ver 8, Applied Maths-Biomerieux, Sint-Martens-Latem, Belgium) evaluation licence that we have received permisson to publish. The dendogram was generated using the similarity coefficients calculated by the number of different bands with 0.5% optimization and a 1% tolerance window for band matching and the clustering method of unweighted pair grouping by mathematical averaging (UPGMA).

Phylogenetic analyses

Phylogenetic analyses were conducted using MEGA X [16]. The evolutionary history was inferred using the maximum likelihood method and Kimura 2-parameter model [17]. The tree with the highest log likelihood was shown. Neighbor-joining and BioNJ algorithms were used to obtain initial trees for the heuristic search and the pairwise distances were estimated using the maximum composite likelihood (MCL) approach. The topology with the superior log likelihood value was selected. Branch lengths were measured based on the number of substitutions per site.

Determination of thermotolerance and ethanol tolerance

To determine temperature and ethanol tolerance, the isolates were first grown in 50 ml enrichment culture (1% glucose [Sigma-Aldrich], 1% yeast extract [Biolife], and 1% ethanol [Isolab]) at 30℃ for 72 h while shaking at 180 rpm [18]. To determine thermotolerance, dilutions of enrichment culture prepared with peptone water were inoculated on glucose, yeast extract, and casein (GYEC) agar (1% glucose [Sigma-Aldrich], 1% yeast extract [Biolife], 2% calcium carbonate [Sigma-Aldrich], and 2% agar [Merck], w/v, containing 4% ethanol [Isolab], v/v, added after autoclaving) [18]. The plates were incubated at different temperatures (30, 35, and 38℃) for 72 h. Ethanol tolerance was also determined on GYEC agar containing different concentrations of ethanol (4, 6, 8, 10, or 12 %, v/v). After inoculation, the plates were incubated at 30℃ for 72 h. The isolates with clear zones were accepted as tolerant to the condition tested. All isolates were analyzed in duplicate.

Determination of cellulose production

Cellulose production of isolates was determined using Hestrin and Schramm (HS) medium (2% glucose [Sigma-Aldrich], 0.5% peptone [Merck], 0.5% yeast extract [Biolife], 0.34% $Na_2HPO_4 \cdot 2H_2O$ [Isolab], and 0.115% citric acid monohydrate, [Merck]; w/v; pH 6.0) [19, 20]. The isolates were inoculated in 5 mL HS medium in duplicate and incubated at 30℃ for 2 d under static conditions. The medium was then transferred to 45 ml HS medium in 250-ml Erlenmeyer flasks and incubated at 30℃ for 7 d under static conditions [19]. The obtained cellulose biofilm was separated from the medium and purified by immersing in 2% NaOH (Sigma-Aldrich) at 80℃ for 45 min and washing in distilled water until it reached pH 7.0. The biofilm was then dried at 45℃ until it reached a constant weight [21].

Results and Discussion

AAB from apricots and vinegars

Among the 69 colonies isolated from apricot and vinegar samples, only 19 were Gram-negative and AAB (Table 1). The remaining isolates were lactic acid bacteria

Table 1. Acetic acid bacteria (AAB) isolates with their temperature and ethanol tolerances.

No	Name	Origin	Isolation medium	Species	Identification Marker	Temperature (℃)			Ethanol concentration (%, v/v)			
						30	35	38	4	6	8	10
1	HFG13	Apricot-Hacihaliloglu	GYC	*Gluconobacter cerinus*	16S rRNA	+	+		+	+		
2	BFG1	Apricot-Hasanbey	GYC	*Gluconobacter cerinus*	16S rRNA	+			+	+		
3	BFG21	Apricot-Hasanbey	GYC	*Gluconobacter cerinus*	GTG5 rep-PCR	+			+	+		
4	BFA14	Apricot-Hasanbey	AAM	*Gluconobacter cerinus*	GTG5 rep-PCR	+	+		+			
5	BFA11	Apricot-Hasanbey	AAM	*Gluconobacter cerinus*	GTG5 rep-PCR	+	+		+			
6	BFA13	Apricot-Hasanbey	AAM	*Gluconobacter cerinus*	16S rRNA	+			+			
7	BFA6	Apricot-Hasanbey	AAM	*Gluconobacter cerinus*	16S rRNA	+			+	+		
8	BFA7	Apricot-Hasanbey	AAM	*Gluconobacter cerinus*	GTG5 rep-PCR	+	+		+			
9	BFA8	Apricot-Hasanbey	AAM	*Gluconobacter cerinus*	GTG5 rep-PCR	+			+			
10	BFA9	Apricot-Hasanbey	AAM	*Gluconobacter cerinus*	16S rRNA	+			+			
11	PS1	Vinegar 1 (Orange)	AAM	*Acetobacter syzygii*	16S rRNA + ITS	+			+	+	+	
12	PS2	Vinegar 1 (Orange)	AAM	*Acetobacter syzygii*	GTG5 rep-PCR	+			+	+		
13	AUS4	Vinegar 2 (red grape)	AAM	*Acetobacter syzygii*	16S rRNA +ITS	+			+	+	+	
14	ELS3	Vinegar 6 (apple)	AAM	*Gluconobacter* sp.	16S rRNA	+			+	+	+	
15	ELS4	Vinegar 6 (apple)	AAM	*Gluconobacter* sp.	16S rRNA + *ligA*	+			+	+		
16	ELS5	Vinegar 6 (apple)	AAM	*Gluconobacter* sp.	16S rRNA + *ligA*	+	+		+	+	+	
17	GUS1	Vinegar 3 (White grape)	AAM	*Komagataeibacter saccharivorans*	16S rRNA + ITS	+	+		+	+	+	
18	GUS2	Vinegar 3 (White grape)	AAM	*Komagataeibacter saccharivorans*	16S rRNA + ITS	+	+		+	+	+	
19	GUS3	Vinegar 3 (White grape)	AAM	*Komagataeibacter* sp.	16S rRNA + ITS	+	+		+	+	+	

Notes: GYC, glucose yeast extract calcium carbonate; AAM, acetic acid medium.

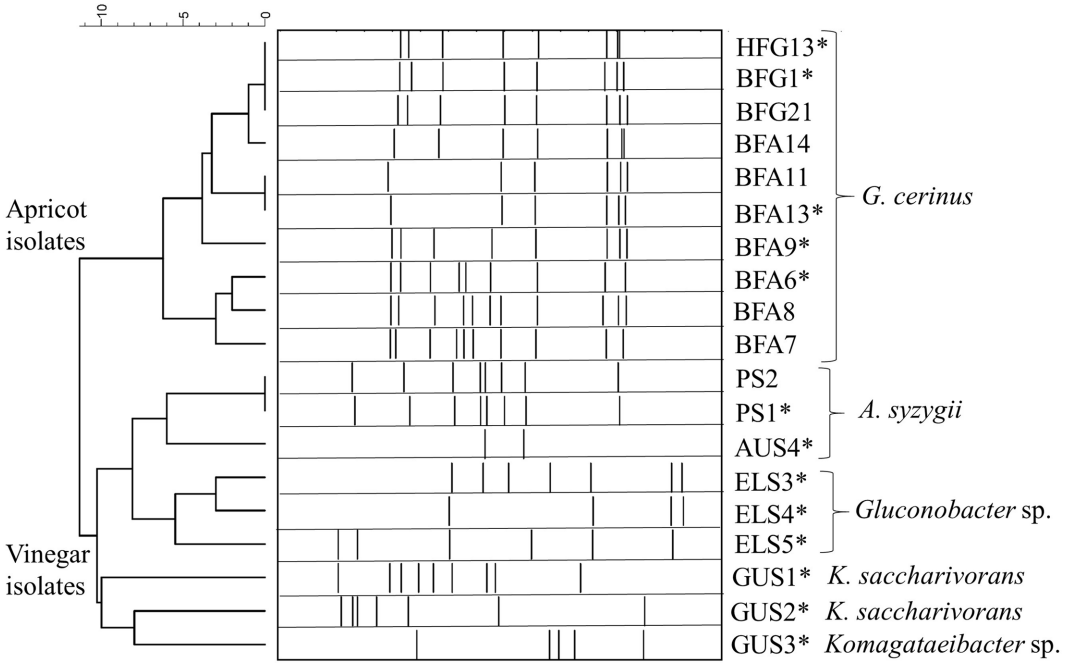

Fig. 1. Dendogram showing the relationship of acetic acid bacteria (AAB) isolates based on their (GTG)5 fingerprinting patterns. Isolates indicated with an asterisk were subjected to sequencing for identification.

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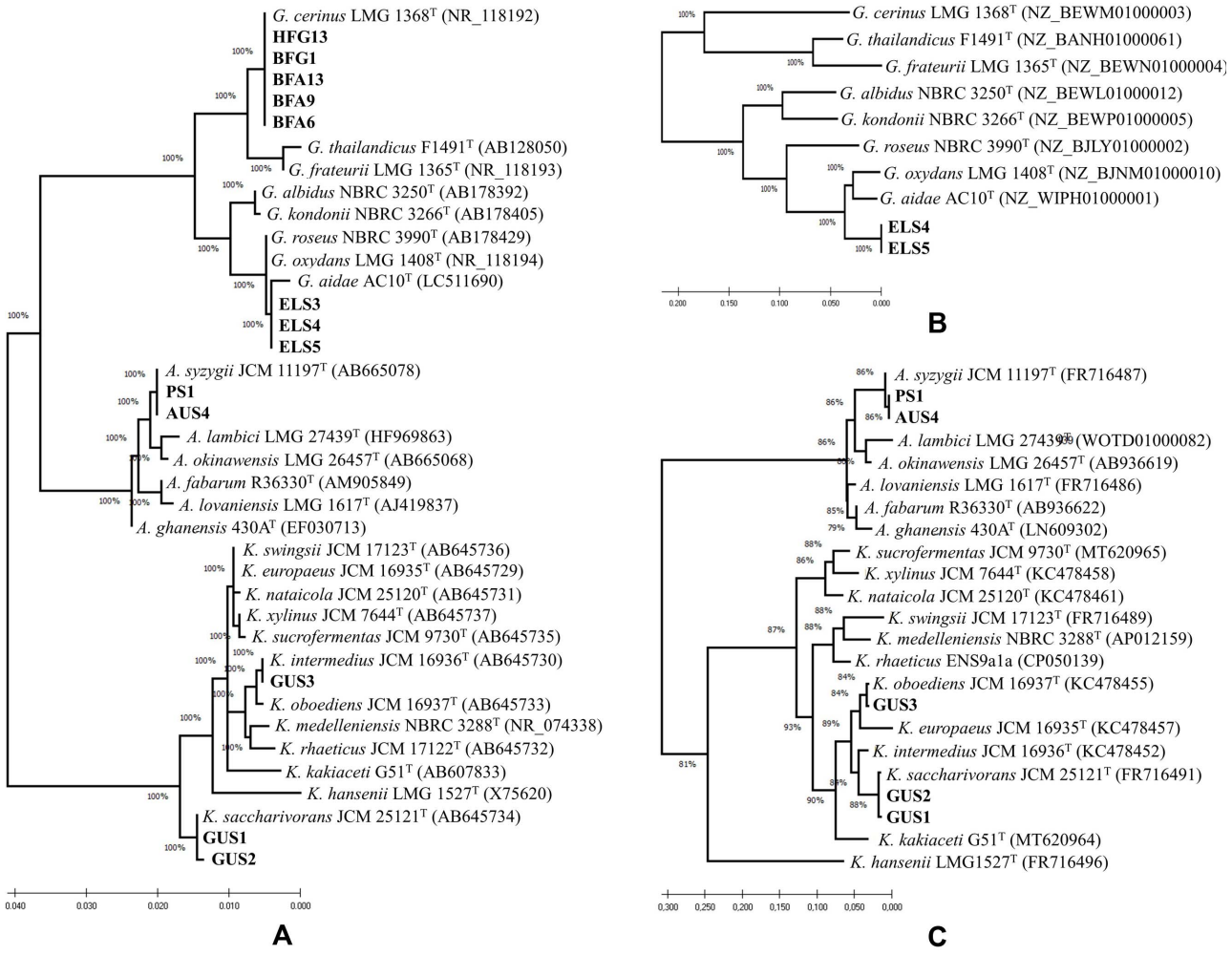

Fig. 2. Phylogenetic relationship of acetic acid bacteria (AAB) isolates based on (a) 16S rRNA (b) *ligA*, and (c) 16S-23S rRNA internal transcribed spacer (ITS) region. 16S rRNA, 16S-23S rRNA ITS and *ligA* analyses involved 1225, 709, and 784 positions, respectively, in the final data set. The l*igA* sequences of the reference strains were extracted from the genome sequences. The GenBank accession numbers are given in parenthesis.

that can grow and produce acid on AAM and GYC agar plates (data not shown).

In the (GTG)5 fingerprinting analyses, the apricot and vinegar isolates were clustered into two different groups (Fig. 1). The selected isolates from each group (indicated by an asterisk in Fig. 1) were subjected to 16S rRNA sequencing. For accurate identification of *Komagataeibacter* and *Acetobacter* isolates, the 16S–23S rRNA ITS region was also used [22–24]. Sequencing results showed that although all apricot isolates were *G. cerinus*, the vinegar isolates were composed of *A. syzygii, K. saccharivorans*, a *Komagataeibacter* sp., and a *Gluconobacter* sp. (Table 1, Fig. 2).

Gluconobacter cerinus is an AAB mainly associated with fruits and flowers [25]. In addition to flowers, it has been isolated from grapes [23] and grape must [26], peach, musk-melon, and a tropical fruit from *Baccaurea* [27]. This is the first study reporting *G. cerinus* from apricots. The presence of *G. cerinus* in both apricot varieties analyzed might be indicative of this species being a frequent microbiota member in these fruits. The different patterns obtained in (GTG)5 fingerprinting implied the presence of several genotypes of *G. cerinus* in the apricots. Similar to our results, Mateo *et al*. [23] have detected *G. cerinus* as the main species in grapes with five different genotypes, as determined by ERIC-PCR

and (GTG)5 fingerprinting techniques, in two vineyards. Navarro *et al*. [26] have also reported four typing profiles of *G. cerinus* revealed by (GTG)5 fingerprinting. In another study, phylogenetic analysis conducted using three housekeeping genes i.e., *dnaK*, *groEL* and *rpoB*, has indicated variability in the *G. cerinus* strains studied [28]. We did not detected differences in the 16S rRNA gene, but recognized several genotypes using (GTG)5 fingerprinting, as shown in Fig. 1. Previous reports [23, 26, 28] in addition to our analyses have indicated a high genetic variability among *G. cerinus* strains.

Gluconobacter sp. ELS3, ELS4, and ELS5 resulted in the same 16S rRNA sequence, which was most closely related to but distinct from the species *G. roseus*, *G. oxydans*, and *G. aidae* (Fig. 2A). To allow better taxonomic positioning, the isolates were subjected to 16S–23S rRNA ITS and *dnaK* PCR; however, multiple banding patterns were observed from agarose gel electrophoresis. One nonspecific PCR product obtained for ELS4 and ELS5 was sequenced and identified as NAD-dependent DNA ligase (*ligA*). *LigA* turned out to be a successful marker for species-level separation (Fig. 2B). In the phylogenetic analysis using *ligA*, the isolates ELS4 and ELS5 separated clearly from *G. oxydans* and *G. aidae*, which suggested that they might represent a new species; this requires further analyses.

For *Komagataeibacter* sp. GUS3, 16S rRNA and 16S–23S rRNA ITS sequencing produced conflicting results. In 16S rRNA sequencing, the isolate appeared to be *K. intermedius* with 100% identity to the JCM 16936^{T}-type species (Fig. 2A); however, in the phylogenetic analyses conducted using 16S–23S rRNA ITS sequences (Fig. 2B), the isolate clustered with the *K. oboediens*-type species LMG 18849^{T} rather than with *K. intermedius*. Additional analyses are needed in future studies to determine the taxonomy of this isolate, which might also represent a different species.

Isolates PS1, PS2, and AUS4 derived from the vinegar samples were identified as *A. syzygii* (Table 1, Figs. 2A, 2C), a species identified in various fermented foods [29, 30], flowers, and fruits [31].

Thermotolerance and ethanol tolerance

AAB are mesophilic bacteria with an optimum temperature of ~30℃; generally, no growth is observed at temperatures >34℃ [32]; however, thermotolerant strains have been reported that can withstand higher temperatures such as 37℃ [33] and even as high as 41–42℃ [34, 35]. Thermotolerance is a desired trait in industrial fermentations to reduce expenses related to cooling [32, 36]. We assessed the temperature tolerance of apricot and vinegar isolates at three, i.e., 30, 35, and 38℃, GYEC agar containing 4% ethanol. The maximum temperature that the isolates tolerated was 35℃ (Table 1). While *A. syzygii* isolates could grow only at 30℃, *Komagataeibacter* species were tolerant up to 35℃. On the other hand, the temperature tolerance of *Gluconobacter* species was strain dependent, and although some could grow only at 30℃, some could withstand up to 35℃. Different high temperature tolerance values have been reported in the literature, even for the same species. For example, *A. syzygii* strains were reported to grow in temperature up to 37℃ and weak growth was observed even at 42℃ [31]. The strain origin as well as the media used to test the tolerance might be factors for strain-level differences. Strains isolated from tropical regions are generally better adapted to higher temperatures [33, 37]. In addition, 4% ethanol addition to yeast extract peptone glycerol glucose medium negatively affects the thermotolerance of *A. pasteurianus* strains compared to that in strains grown without adding ethanol [34].

Ethanol tolerance is a useful trait for high-efficiency vinegar production because the initial high ethanol concentration might serve as an important stressor affecting the growth and acidification ability of AAB [38]. The ethanol tolerance of the isolates was screened using four ethanol concentrations 4, 6, 8, and 10%. Although the *G. cerinus* isolates originating from apricot samples were resistant to 4–6% ethanol, the vinegar isolates were more resistant up to 8% ethanol. This makes sense considering the higher ethanol level of vinegar compared to that in fruits.

Bacterial cellulose production

Bacterial cellulose is a versatile biopolymer that has applications in the food, biomedical, cosmetic and various engineering industries [4]. Among all the isolates tested, only *Komagataeibacter* sp. GUS3 produced cellulose. The production level was ~1 g/l (0.993 ± 0.007 g/l) at 30℃ in a 7-d incubation period in HS medium under static conditions. This value is promising and might be

further increased by optimizing cultivation conditions and media components in future studies.

In conclusion, in the present study, 19 AAB were isolated from the Malatya apricot (PDO) and various homemade fruit vinegar samples. Molecular identification of the isolates was conducted using 16S rRNA alone or in combination to 16S–23S rRNA ITS or *ligA*. Although it is an unintended unspecific amplification product, *ligA* separated species boundaries well in phylogenetic analysis, which indicated that it could be used as a phylogenetic marker for AAB. The only AAB species obtained from the Malatya apricot was *G. cerinus*. The isolation of this species in two different varieties, Hacihaliloglu and Hasanbey, demonstrated that *G. cerinus* might be a frequent AAB member in the Malatya apricot. (GTG)5 fingerprinting showed that several *G. cerinus* genotypes were present in the samples. The vinegar isolates were composed of *A. syzygii* and *K. saccharivorans* and two possibly new species, *Komagataeibacter* sp. and *Gluconobacter* sp. The highest temperature the isolates tolerated was 35℃. Vinegar isolates were generally more resistant to ethanol concentration at up to 8%, compared to the apricot isolates. One isolate, *Komagataeibacter* sp. GUS3, produced bacterial cellulose at ~1 g/l in HS medium in 7 d under static conditions, which might be increased using optimization studies.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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