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ORIGINAL ARTICLE

MALDI-TOF/TOF mass spectrometry for determination of yeast diversity in traditional cornelian cherry tarhana produced with different cereal/pseudocereal flours

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Abstract

Purpose This study is to determine the dynamics in the microflora of cornelian cherry tarhana (CCT), a traditional food of Turkey, by MALDI-TOF/TOF MS.

Methods Non-fermented and fermented CCT productions were performed by using flours of bread wheat, wholegrain hull-less barley, buckwheat, and clear flour. Identification of the isolates obtained from raw materials and various production steps of the CCT was performed by MALDI-TOF/TOF MS. Dendograms were prepared by cluster analysis and the distances between the strains isolated during production stages and from cornelian cherry puree were determined.

Results Totally, 231 isolates were obtained and 45.5% of them were identified at species level, 30.3% at genus level while 24.2% of the isolates could not be identified. It was found that microflora of cornelian cherry tarhana is mainly composed of yeasts. Thirty-two percent of the identified yeast isolates were *Hanseniaspora uvarum* and the others were *Saccharomyces cerevisiae* (19.6%), *Torulaspora delbrueckii* (18.6%), *Candida krusei* (11.3%), *Candida lusitaniae* (9.3%), *Metschnikowia pulcherrima* (3.1%), *Wickerhamomyces anomalus* (2.1%), *Candida kefyr* (2.1%), *Cyberlindnera fabianii* (1%), and *Candida parapsilosis* (1%). Only two lactic acid bacteria could be isolated, which were *Lactobacillus reuteri* and *Enterococcus* spp., originating from buckwheat flour and clear flour, respectively. Dendograms revealed that some of the yeast strains isolated during production were originating from cornelian cherry.

Conclusions Microflora of CCT was investigated for the first time. This is one of the few studies using MALDI-TOF/TOF MS for identification of food originated yeasts. Novel species–identified, endogenic yeasts which could have potential technological characteristics were introduced.

Keywords Cornelian cherry tarhana · Yeast · Lactic acid bacteria · MALDI-TOF/TOF MS

Introduction

Fermented foods and beverages were among the first processed food products consumed by humans. Fermentations could be described based on microorganisms involved, such as yeast, lactic acid bacteria (LAB), and acetic acid bacteria, and based on food substrates, which include meats and fish, dairy, fruits and vegetables, legumes, and cereals. Due to the rich variety of food-microorganism combinations, there are thousands of national or international fermented foods and beverages (Marco et al. 2017). Among the cereal-based fermented foods, bread and beer are the most popular ones. But, there are also many other products prepared in different regions of the world (tempeh, pito, sekete, kwass and etc.) and in Turkey (boza, tarhana, kumru, chickpea bread, and etc.). Most of these products are manufactured in homes, villages, and/or small-scale industries by using either wheat, maize, barley, oat, millet, rice, or their flours (Tanguler et al. 2014).

Tarhana is an old and popular traditional Turkish fermented product which is generally consumed as soup. It is produced mainly by mixing wheat flour, yoghurt, yeast, and a variety of cooked vegetables and spices (tomato, onion, salt, mint, paprika), followed by fermentation, drying, and grinding (Ozdemir et al. 2007; Tanguler et al. 2014). According to the Turkish Standardization Institute, there are four categories of

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tarhana based on the production method employed, which are "flour tarhana," "goce tarhana", "semolina tarhana," and "mixed tarhana." The use of wheat flour, chopped wheat, and semolina separately or as combinations in the recipe makes them different (Kabak and Dobson 2011). However, there are also various studies which other cereal and legume flours such as barley, buckwheat, corn, lupin, and rice are used in the production of tarhana. On the other hand, some plant and fruits such as cherry laurel, bilberry fruit, grape, and cornelian cherry could be added to formulation of traditional wheat flour tarhana (Cagindi et al. 2016).

Cornelian cherry tarhana, also named as sour tarhana, is traditionally produced in a few number of provinces including Bolu, and also having a geographical mark in Bolu. The major distinction of the CCT is that yoghurt is not used in the tarhana formulation. Additionally, the CCT productions are usually performed without fermentation, except one type of the CCT produced in Kutahya province. Briefly, the tarhana dough is prepared by mixing of wheat flour and raw or cooked cornelian cherry or its puree at a certain ratio. Kneading is applied, followed by drying. There are only a few studies about CCT, because it is domestic and produced in a limited area. On the other hand, so many studies could be found in the literature about the phytochemicals and their chemical structures and also pharmacological activities of the cornelian cherry (Dinda et al. 2016).

Every traditional fermented food has a specific microflora and fermented foods serve as a good source of probiotic and functional microorganisms which could have important physiological properties. Tarhana fermentation is accomplished with LAB and yeasts and there are several reported studies about determination of tarhana microflora (Sengun et al. 2009; Settanni et al. 2011; Ozel et al. 2015; Simsek et al. 2017). However, there is no reported study about the microflora of CCT. In this study, CCT was produced traditionally and also by applying fermentation. Productions were performed by using flours of bread wheat, wholegrain hull-less barley, buckwheat, and clear flour. Fermentation was applied for the first time and it was aimed to determine the effects of fermentation as well as using different raw materials on the microflora. Matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) was used for microbial identifications. Similarity levels between the isolates of the same species were determined by using the dendrograms.

Materials and methods

Raw materials

For the production of cornelian cherry tarhana (CCT), flours of bread wheat, hull-less barley, buckwheat, and clear flour (a by-product of semolina industry), uncooked cornelian cherry puree, and salt were used as ingredients. Hull-less barley (*Ozen cv.*) was provided from the Central Research Institute of Field Crops (Ankara, Turkey) and grinded to wholegrain hull-less barley flour by using stone mill (A700 Genuine Wood, Good Mills Company, Osttirol, Lienz, Austria). Buckwheat (*Gunes cv.*) was obtained from the Bahri Dagdas International Agricultural Research Institute (Konya, Turkey) and it was grinded to buckwheat flour by using laboratory roller-mill (Quadrumat Junior, Brabender GmbH & Co., Duisburg, Germany). Clear flour was supplied from the semolina milling unit of a local pasta company. The cornelian cherry (*Cornus mas* L.) was obtained from local producers in the Bolu province. Cornelian cherry puree was prepared in the production plant of local company in Bolu.

Cornelian cherry tarhana production

Productions of CCT by using bread wheat (traditional), wholegrain hull-less barley, and buckwheat flours and clear flour were performed in one of the company's production plant. CCT samples were produced also by applying fermentation, besides using traditional production procedure without fermentation. The formulation of the cornelian cherry tarhana doughs and production steps were determined according to the proposals of the CCT producers. Traditional CCT was produced by using bread wheat flour [puree (52.7%), flour (42%), salt (5.3%)], wholegrain hull-less barley flour [puree (59.4%), flour (34.7%), salt (5.9%)], buckwheat flour [puree (55.3%), flour (39.2%), salt (5.5%)], and clear flour [puree (58%), flour (36.2%), salt (5.8%)]. For each of the formulation, the ingredients were mixed in the specified quantities and homogenously kneaded (KitchenAid, USA) to make tarhana doughs in similar dough consistencies.

The tarhana dough samples were spread on the separate clothes in the portions of approximately 20 g and dried at 25 °C and 60% humidity for 2 days. The dried products were grinded and they were left to final drying for 6 days under the same conditions. At the end of this period, the CCT samples produced from the different kinds of flours were again grinded to obtain the final non-fermented products.

For preparing the fermented tarhana samples, fermentation process was applied to the kneaded tarhana doughs in covered boxes at 25 °C for 3 days. Fermentation step was performed without adding any starter culture. After the fermentation step, drying, grinding, and the final drying procedures were applied at the same conditions as used for the non-fermented products. The non-fermented and fermented products were taken into polyethylene bags and they were stored in the refrigerator conditions until analysis.

Sampling procedure

Samples from raw materials and different production steps were taken to investigate the changes in the yeast and lactic acid bacteria (LAB) microflora of the non-fermented and fermented CCT products. The samples including tarhana dough, dried product, and final product were taken from the non-fermented tarhana. Additionally, samples at the 24th, 48th, and 72nd hours of the fermentation process were taken from the fermented tarhana. After the sampling, the isolation and identification of the microorganisms were carried out.

Isolation of yeast and LAB

Twenty-five grams of samples were homogenized in 225 mL of 0.1% sterile peptone water, then serial dilutions were prepared. For yeast enumeration and isolation, each dilution was spread on YGC (yeast extract glucose chloramphenicol) agar plates composed of (g/L): yeast extract, 5; glucose, 20; chloramphenicol, 0.1; and agar, 14.9. The number of yeasts was determined and the different colony morphologies were selected from countable plates, following the incubation at 28 °C for 48 h. Cultures were activated in YM (yeast extract malt extract) broth and microscopic morphologies of the yeasts were examined. The colonies were purified by re-streaking on the YM (yeast extract malt extract) agar. Cultures were stored at 4 °C in YM agar and activated in the same medium.

LAB isolation was performed by using MRS and M17 agar. The plates for MRS agar were incubated under microaerophilic conditions and the M17 agar plates were incubated aerobically at 37 °C for 24–48 h. The colony morphologies were observed and the different ones were purified on their isolation medium (MRS or M17) agar. Purified cultures were stored in 20% glycerol at − 20 °C and the stock cultures were cultivated for further experiments. Additionally, Gram staining and catalase test were applied to the isolates from MRS and M17 agar. At this stage, Gram-positive and catalase-positive cultures were selected and passed on the subsequent step.

Identification by MALDI-TOF/TOF MS

In this study, identifications of the isolated lactic acid bacteria and yeast cultures were performed by using MALDI-TOF/TOF MS in Scientific Industrial and Technological Application and Research Center of Bolu Abant Izzet Baysal University, Turkey. Direct transfer method, direct transfer-formic acid method, and extraction method were used for the identification of the microorganisms with the Bruker MALDI-TOF/TOF MS (Autoflex Speed Bruker Daltonics, Germany). The direct transfer method was described by Schulthess et al. (2013) as transferring of fresh colony onto target plate's spot and being overlaid with 4-hydroxy-α-cyanocinnamic acid ("alphacyano" or 4-HCCA) matrix. However, majority of the isolates could not be identified with this method; direct transfer-formic acid method was carried out, which 70% formic acid was added to spots before the matrix solution. The considerable majority of the microorganisms were identified by using this given method. To identify the unknown cultures, another proposed extraction procedure was followed. In this method, a fresh colony was suspended in deionized water and ethanol was added; and after centrifugation of the cell suspension, the supernatant was discarded and the pellet was resuspended in 70% formic acid and acetonitrile. Following the second centrifugation, the supernatant was transferred to the spot before being overlaid with 4-HCCA matrix (Schulthess et al. 2013).

Mass spectra were processed using BioTyper software (version 3.1; Bruker Daltonics). As reported by Nacef et al. (2017), matching between experimental profiles obtained from the microorganism isolates and the reference profiles is expressed by BioTyper according to a score. It was briefly explained that if a score is higher than 2.0, it means identification at the species level and the value between 1.7 and 2.0 imply only genus identification. On the other hand, the score value under 1.7 shows no significant similarity between the unknown profile and the database (Nacef et al. 2017). Cluster analysis was performed for MALDI-TOF/TOF main mass spectra (MSP) of yeast strains originated from cornelian cherry puree and from different kinds of cornelian cherry tarhana. Dendograms of identified strains were generated with the Biotyper MSP Dendogram Creation Standard Method. The mass spectra of common yeast species (*H. uvarum*, *C. lusitaniae*, *C. krusei*, and *S. cerevisiae*) which were originated from cornelian cherry puree were chosen for the dendograms. Four different dendograms were prepared for each CCT production. Each of the dendogram indicated the distance level between cornelian cherry puree strains and the others from different stages of CCT production. It was aimed to screen a yeast strain obtained from any production stage.

Results

A total of 231 isolates were obtained at the production stages of the non-fermented and fermented CCT samples made from cornelian cherry and four different kinds of flours. As an outcome of the identification by using MALDI-TOF/TOF MS, 161 of the total 231 microorganisms were determined to be yeasts and species (97) and genus (64) identifications of them were carried out. Fourteen of the isolates were detected to be in different genera of bacteria including *Bacillus* (11), *Kosakonia* (1), *Enterococcus* (1), and *Lactobacillus* (1). However, identification of 56 isolates could not be possible, corresponding to 24.2% of total number. Overall analyzing

results indicated that 45.5% and 30.3% of the isolates were identified at species and genus levels, respectively.

The yeast counts of the cornelian cherry puree, wholegrain hull-less barley, and buckwheat flours and clear flour were found as 6.36, 4.30, 4.61, and 3.70 log cfu/g, respectively. However, yeast growth could not be observed in the wheat flour. The yeast counts of the four non-fermented tarhana doughs were found to change between 4.81 and 5.14 log cfu/g and these values were in the range of 3.00–3.54 log cfu/g for the final products of CCT. On the other hand, it was clearly observed that the fermentation process leads to an increase in the yeast counts of the tarhana doughs. The tarhana doughs contained fewer amounts of yeasts (4.46–5.62 log cfu/g) at the beginning, compared to the doughs at the 72nd hours of the fermentation (6.24–6.38 log cfu/g). The yeast counts decreased after the drying stages of fermented CCT products, similarly to the non-fermented CCT (data not shown).

The obtained MALDI-TOF/TOF MS profiles of the isolates were compared to the reference spectra of the BioTyper database for their similarity, and then, the isolates were identified at species or genus levels regarding to the given BioTyper score values. Identification results obtained during CCT productions were given in Table 1. Eight isolates of cornelian cherry puree were identified as *Hanseniaspora uvarum* (4), *Saccharomyces cerevisiae* (1), *Candida*

Table 1 Identification results of the isolates obtained by MALDI-TOF/TOF MS

Raw materials/products	Identified isolates
Cornelian cherry puree	*H. uvarum* MP-1, MP-2, MP-3, MP-5; *S. cerevisiae* LP-6; *C. lusitaniae* LP-7, LP-9; *C. krusei* LP-2; **S. cerevisiae* LP-8; **C. krusei* LP-11
Buckwheat flour	*L. reuteri* LKBU-4; *K. cowanii* LKBU-3
Clear flour	*C. fabianii* MIAU-1; **C. fabianii* LIAU-6; **Enterococcus faecium* LIAU-2
Non-fermented CCT produced with wheat flour	*H. uvarum* MKH-1, MKD-2; *T. delbrueckii* MKH-3, MKD-1; *S. cerevisiae* MKH-2, MKH-4, LKH-3; *M. pulcherrima* MKD-3; *C. krusei* LKH-1; *C. lusitaniae* LKH-4; **C. lusitaniae* LKH-6;**C. krusei* LKD-1, LKD-5, LKD-6
Fermented CCT produced with wheat flour	*H. uvarum* MFKHc-2, MFKD-2; *T. delbrueckii* MFKS-1, MFKS-2; *M. pulcherrima* MFKD-1; *C. krusei* LFKH-1, LFKHc-1, LFKD-2; **C. lusitaniae* MFKH-1, LFKH-6; **C. krusei* LFKH-7; **M. pulcherrima* MFKH-3, MFKHa-1, MFKHb-3, MFKS-3;**S. cerevisiae* LFKHc-6; **P. kluyveri* LFKH-5
Non-fermented CCT produced with wholegrain hull-less barley flour	*H. uvarum* MAH-1; *S. cerevisiae* MAH-5, MAD-1, MAD-2, LAH-4, LAH-8; *T. delbrueckii* MAH-3, MAD-3; *C. lusitaniae* LAH-6, LAD-1;**S. cerevisiae* MAH-4, MAD-5; **M. pulcherrima* MAD-4
Fermented CCT produced with wholegrain hull-less barley flour	*H. uvarum* MFAH-2, MFAHa-1, MFAHb-3, MFAHc-2; *T. delbrueckii* MFAHa-2, MFAS-1; *M. pulcherrima* MFAHc-1; *S. cerevisiae* LFAD-5; *C. kefyr* LFAH-6; **M. pulcherrima* MFAH-1, MFAHa-4, MFAHb-2, MFAD-2, MFAS-2; **C. lusitaniae* LFAHb-6, LFAHb-1, LFAD-7, LFAS-2; **C. krusei* LFAHb-5, LFAD-6; **C. guilliermondii* LFAHa-2; **P. kluyveri* MFAHa-3, MFAHb-1; **S. cerevisiae* LFAD-4
Non-fermented CCT produced with buckwheat flour	*H. uvarum* MKBH-4, MKBD-2; *S. cerevisiae* MKBH-1, MKBD-4, LKBH-6; *T. delbrueckii* MKBH-5, MKBD-1; **M. pulcherrima MKBD-3*;**S. cerevisiae* LKBD-9, LKBD-2, LKBD-7; *B. thuringiensis* LKBH-4
Fermented CCT produced with buckwheat flour	*H. uvarum* MFKBH-2, MFKBHa-2-1, MFKBHa-4, MFKBHb-3, MFKBHc-2; *T. delbrueckii* MFKBH-1; *W. anomalus* MFKBHb-2; *S. cerevisiae* MFKBD-3, LFKBHc-1; *C. krusei* LFKBHa-8, LFKBHc-8, LFKBD-3; *C. lusitaniae* LFKBHa-4; *C. kefyr* LFKBH-9; **C. krusei* MFKBHa-1; **C. lusitaniae* LFKBH-6, LFKBH-8, LFKBHc-2;**C. valida* MFKBHa-2-2; **M. pulcherrima* MFKBHa-3, MFKBHb-1, MFKBHc-1, MFKBD-2, MFKBD-4, MFKBS-1; **S. cerevisiae* LFKBHa-7, LFKBHb-10, LFKBD-1; **H. uvarum* MFKBD-1; **Bacillus pumilus* LFKBH-1; **B. licheniformis* LFKBS-2
Non-fermented CCT produced with clear flour	*H. uvarum* MIAH-1, MIAH-3, MIAH-7, MIAD-1; *S. cerevisiae* MIAH-5, MIAH-6, MIAH-8, LIAH-1; *T. delbrueckii* MIAH-2, MIAD-2, MIAS-1, MIAS-4; *C. lusitaniae* LIAH-10; **M. pulcherrima* MIAD-3; **C. lusitaniae* LIAD-8; *B. oleronius* LIAH-6; **B. cereus* LIAD-5
Fermented CCT produced with clear flour	*H. uvarum* MFIAH-3, MFIAH-5, MFIAHa-1, MFIAHb-1, MFIAHc-1, MFIAHc-2, MFIAD-2; *T. delbrueckii* MFIAHa-4, MFIAHb-2, MFIAS-3; *C. krusei* MFIAH-1, LFIAHb-9, LFIAH-4; *W. anomalus* MFIAHb-4; *C. parapsilosis* LFIAHb-8; *C. lusitaniae* MFIAH-6, LFIAH-5; **C. valida* MFIAH-8; **C. lusitaniae* LFIAHc-2, LFIAHc-5, LFIAD-4; **M. pulcherrima* MFIAHa-2, MFIAHb-3, MFIAS-2; **W. anomalus* MFIAD-1; * *S. cerevisiae* LFIAHb-4; *B. cereus* LFIAH-1, LFIAHc-7; *B. licheniformis* LFIAH-3, LFIAD-6; **B. pumilus* LFIAHa-1; **Bacillus circulans* LFIAS-1

*Identified at genus level

lusitaniae (2), and *Candida krusei* (1). Furthermore, two isolates were identified at genus level which were *S. cerevisiae* and *C. krusei*. As an example, mass spectra of the isolates *H. uvarum* MP-1 and *S. cerevisiae* LP-6 were given in Fig. 1. In addition, one isolate was identified as *Lactobacillus reuteri* and one isolate of *Kosakonia cowanii* was encountered in buckwheat flour. In clear flour, two yeast strains belonged to *Cyberlindnera fabianii* (at levels of 1 species and 1 genus) and one LAB was identified as *Enterococcus* spp. Furthermore, no isolates could be obtained from the flours of wheat and hull-less barley.

For the CCT with wheat flour, 10 isolates at species level (*H. uvarum*, 2; *S. cerevisiae*, 3; *Torulaspora delbrueckii*, 2; *Metschnikowia pulcherrima*, 1; *C. krusei*, 1; and *C. lusitaniae*, 1) and 4 *Candida* spp. were identified (Table 1). However, 6 of the total 20 isolates could not be identified (score ≤ 1.7). In fermented CCT with wheat flour, 17 of the total 31 isolates could be identified and based on the species-level identification (8) results, the isolates were *H. uvarum* (2), *T. delbrueckii* (2), *M. pulcherrima* (1), and *C. krusei* (3). In addition, 9 of the 17 isolates were identified at genus level and they were given as *Candida* (3), *Metschnikowia* (4), *Pichia* (1), and *Saccharomyces* (1).

With regard to the identification results of the isolates obtained from the non-fermented CCT with wholegrain hull-less barley flour, 13 of the total 16 yeast isolates were identified. Ten of the isolates belonged to species of *H. uvarum* (1), *S. cerevisiae* (5), *T. delbrueckii* (2), and *C. lusitaniae* (2), while the other three isolates were in the genera of *Saccharomyces* (2) and *Metschnikowia* (1). For the fermented CCT with wholegrain hull-less barley flour, 9 yeast isolates (*H. uvarum*, 4; *T. delbrueckii*, 2; *M. pulcherrima*, 1; *S. cerevisiae*, 1; *Candida kefyr*, 1) were identified at species level. Besides, genera of 15 isolates including *Candida* (7), *Metschnikowia* (5), *Pichia* (2), and *Saccharomyces* (1) were performed and seven of the total 31 isolates could not be identified (Table 1).

For the non-fermented CCT with buckwheat flour, species identifications of 8 of the 12 isolates were achieved. Seven of the identified strains were yeasts (*H. uvarum*, 2; *S. cerevisiae*, 3; and *T. delbrueckii*, 7) and one of them was bacteria (*Bacillus thuringiensis*). Four isolates were identified at genus level as *Metschnikowia* (1) and *Saccharomyces* (3). However 4 isolates could not be identified. In the fermented CCT with buckwheat flour, 31 of the total 38 isolates were identified. The species of the 14 yeasts were found as *H. uvarum* (5), *T. delbrueckii* (1), *Wickerhamomyces anomalus* (1), *S. cerevisiae* (2), *C. krusei* (3), *C. lusitaniae* (1), and *C. kefyr* (1). Furthermore, 15 yeast isolates were in the genera of *Candida* (5), *Metschnikowia* (6), *Hanseniaspora* (1), and *Saccharomyces* (3) and two bacteria isolates were identified as *Bacillus* spp.

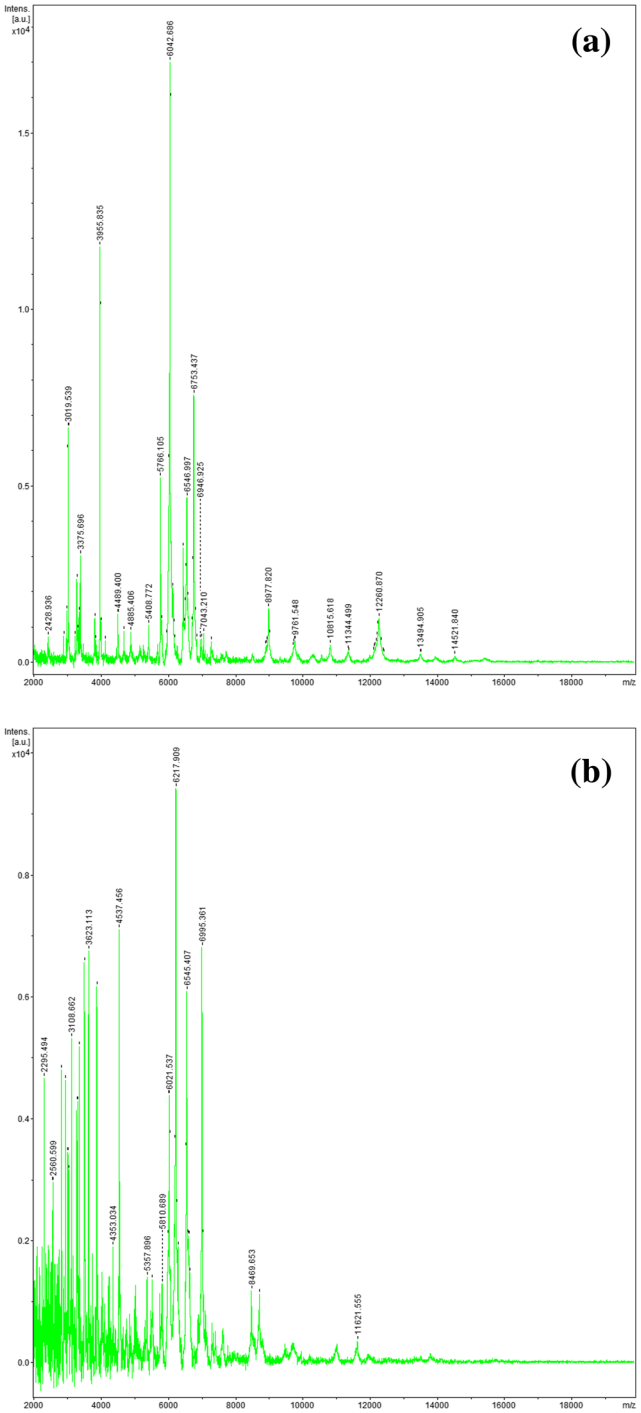


Fig. 1 MALDI-TOF/TOF mass spectra of strains *H. uvarum* MP-1 (**a**) and *S. cerevisiae* LP-6 (**b**)

When the identification results of the non-fermented CCT with clear flour were investigated, it could be revealed that 17 of the total 20 isolates were identified. Fourteen of the isolates were identified at species level as *H. uvarum* (4), *S. cerevisiae* (4), *T. delbrueckii* (4), *C. lusitaniae* (1), and *Bacillus oleronius* (1), in addition to genus identifications as *Metschnikowia* (1), *Candida* (1), and *Bacillus* (1). For the fermented CCT with clear flour, 21 of the 32 isolates were identified at species

level, 17 of which were yeasts (*H. uvarum*, 7; *T. delbrueckii*, 3, *C. krusei*, 3; *W. anomalus*, 1; *Candida parapsilosis*, 1 and *C. lusitaniae*, 2) and four of them were bacteria (*Bacillus cereus*, 2; *Bacillus licheniformis*, 2). The genus identifications were as Candida (4), *Metschnikowia* (3), 1 *Wickerhamomyces* (1), *Saccharomyces* (1), and *Bacillus* (2) and 12 of the total 44 isolates could not be identified (Table 1).

Identified (161) yeast isolates were in seven different genera and their percentage could be summarized as the following: *Candida*, 30.4%; *Hanseniaspora*, 19.9%; *Saccharomyces*, 19.3%; *Metschnikowia*, 14.9%; *Pichia*, 1.9%; *Wickerhamomyces*, 1.9%; and *Cyberlindnera*, 1.2%. Ninety-seven isolates were identified at species level and belonged to ten different species which were *H. uvarum* (32%), *S. cerevisiae* (19.6%), *T. delbrueckii* (18.6%), *C. krusei* (11.3%), *C. lusitaniae* (9.3%), *M. pulcherrima* (3.1%), *W. anomalus* (2.1%), *C. kefyr* (2.1%), *Cy. fabianii* (1%), and *C. parapsilosis* (1%). Although the microflora of CCT samples was dominated by *Candida* genus (30.4%), the mostly identified species was found to be *H. uvarum*.

Results of species identifications in accordance with different production stages were given in Figs. 2 and 3. Since the microflora of CCT samples was determined to be yeast dominated, only the identified yeast species were included in these figures. The identification results of the non-fermented and fermented CCT produced from wheat flour were shown in Fig. 2. *S. cerevisiae* was determined to be the main species in the dough of the non-fermented CCT with wheat flour, as well as *H. uvarum*, *C. lusitaniae*, *C. krusei*, S. *cerevisiae*, and *T. delbrueckii*. However, *H. uvarum* and *T. delbrueckii* along with *M. pulcherrima* could be isolated after the first drying of the non-fermented tarhana. For the fermented CCT with wheat flour, *C. krusei* was the only species to be isolated in dough. Furthermore, the fermentation process stimulated the growth of *H. uvarum*, *M. pulcherrima*, and *Pichia* spp. Although no yeast was identified after the final drying of the non-fermented product, *T. delbrueckii* was the only species isolated from the fermented product even after the final drying.

The predominant species was observed to be *S. cerevisiae* for non-fermented CCT produced with wholegrain hull-less barley flour as shown in Table 1. *H. uvarum*, *C. lusitaniae*, and *S. cerevisiae* species isolated from cornelian cherry puree as well as *C. kefyr* were also encountered in the tarhana dough (Fig. 2). However, only *C. lusitaniae* and *S. cerevisiae* as well as *T. delbrueckii* could be identified after the first drying of the non-fermented tarhana. Fermentation process stimulated the growth of *M. pulcherrima* and *Pichia* spp. The mostly isolated species *S. cerevisiae* in the non-fermented product did not appear until the first drying stage of the fermented tarhana. *T. delbrueckii* was isolated only from the final product of the fermented tarhana. *T. delbrueckii* was unexpectedly isolated from the dried and final products of CCT with wholegrain hull-less barley flour, although it could not be detected in the cornelian cherry and other stages of the production.

The results of species identification and isolation sources of the yeasts for CCT with buckwheat flour could be seen in Fig. 3. *S. cerevisiae* was the main species found in the non-fermented tarhana similarly to the non-fermented CCT with wheat and wholegrain hull-less barley flour. However, the predominant species were *H. uvarum* and *C. krusei* for the fermented CCT. The species of *S. cerevisiae*, *H. uvarum*, and *T. delbrueckii* were isolated from the dough and first dried product of the non-fermented tarhana. In the fermented CCT with buckwheat flour, *H. uvarum* probably originated from cornelian cherry was isolated at all time points of fermentation besides tarhana dough. The growth of *C. krusei* had been promoted by fermentation and it was also isolated after the first drying of the fermented CCT. *W. anomalus* was the distinct species which was only detected in the 48th hours of the fermentation.

In the production of non-fermented CCT with clear flour, *S. cerevisiae*, *H. uvarum*, and *T. delbrueckii* were observed as predominant species. As could be seen from Fig. 3, the species *H. uvarum*, *C. lusitaniae*, and *S. cerevisiae* belonging to cornelian cherry were also isolated from non-fermented tarhana dough. However, only *H. uvarum* could be observed in dried tarhana. Species of *T. delbrueckii* was isolated from the dough and final product of the non-fermented tarhana. For the fermented tarhana, it was emphasized that *H. uvarum*, which was isolated from dough, survived during the fermentation process similarly to other fermented products. *H. uvarum* was detected also to be predominant species for the fermented CCT with clear flour. Besides other yeasts, *W. anomalus* was isolated at the 48th hours of the fermentation, similar to the fermented buckwheat flour tarhana.

In this study, in addition to majority of yeasts, a few of bacteria were also isolated in CCT products with buckwheat flour and clear flour. For buckwheat flour tarhana, these isolates were obtained from dough of the non-fermented and fermented buckwheat tarhana and they were identified as *B. thuringiensis* and *Bacillus* spp., respectively. One of the *Bacillus* spp. was also isolated from the final product of the fermented CCT with buckwheat flour. In addition, *Bacillus oleronius* and *Bacillus* spp. were originated from the dough and final product of the non-fermented tarhana, in addition to *B. cereus*, *B. licheniformis*, and *Bacillus* spp. which were isolated from production stages of the fermented CCT produced with clear flour.

The distances between the isolates from cornelian cherry puree and CCT with wheat flour were demonstrated by the dendogram in Fig. 4. It could be seen that *C. lusitaniae* LKH-4 was very close to puree isolates (LP7 and LP9) indicating that this strain isolated from tarhana dough was originated from cornelian cherry puree. *C. krusei* LP-2 strain of cornelian cherry puree was determined to be close to (0.2) *C. krusei* LKH-1 which was isolated from non-fermented tarhana dough. However, strains of *C. krusei* LFKH-1, LFKD-2, and LFKHc-1 obtained

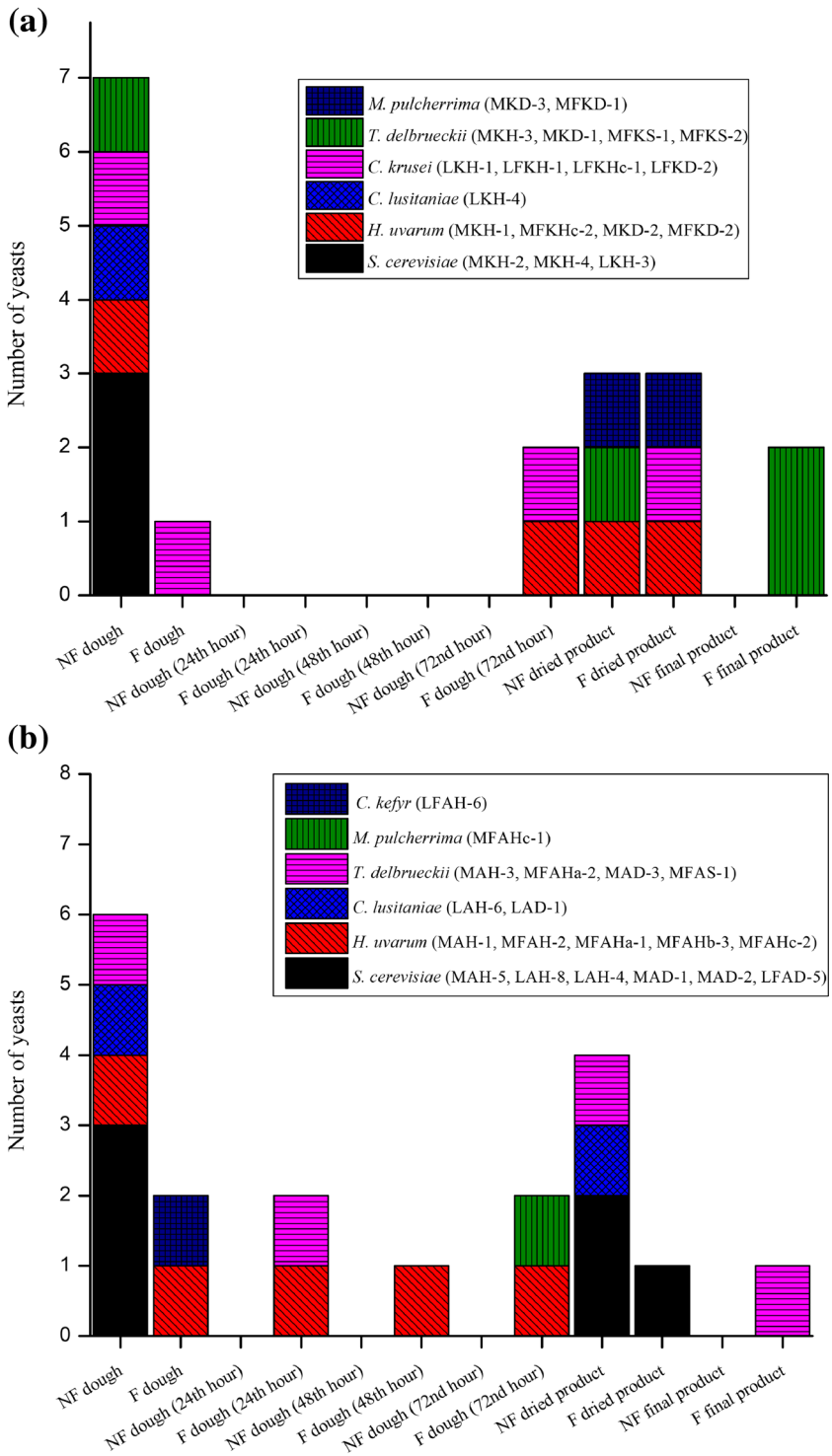


Fig. 2 Identified yeast species from different production steps of the CCT produced with wheat flour (**a**) and wholegrain hull-less barley flour (**b**) (F, fermented; NF, non-fermented)

from the fermented tarhana clustered as a distinct branch and they were found to be close to each other. *S. cerevisiae* strains encountered in tarhana dough (MKH-2 and MKH-4) were relatively distinct from (0.5) *S. cerevisiae* LP-6. As it was shown, *H. uvarum* strains obtained from cornelian cherry puree were distinct from particularly the strain of MFKD-2 (0.7) indicating that its source is probably different from puree.

It was observed from Fig. 4 that *C. lusitaniae* LAH-6 and LAD-1 strains isolated from the non-fermented hull-less barley flour tarhana dough and dried tarhana were probably also encountered in cornelian cherry puree, as they were very close to LP-9 and LP-7 strains. All *H. uvarum* strains of cornelian cherry puree and the strain MAH-1 originating from the non-fermented tarhana dough clustered together with all fermented

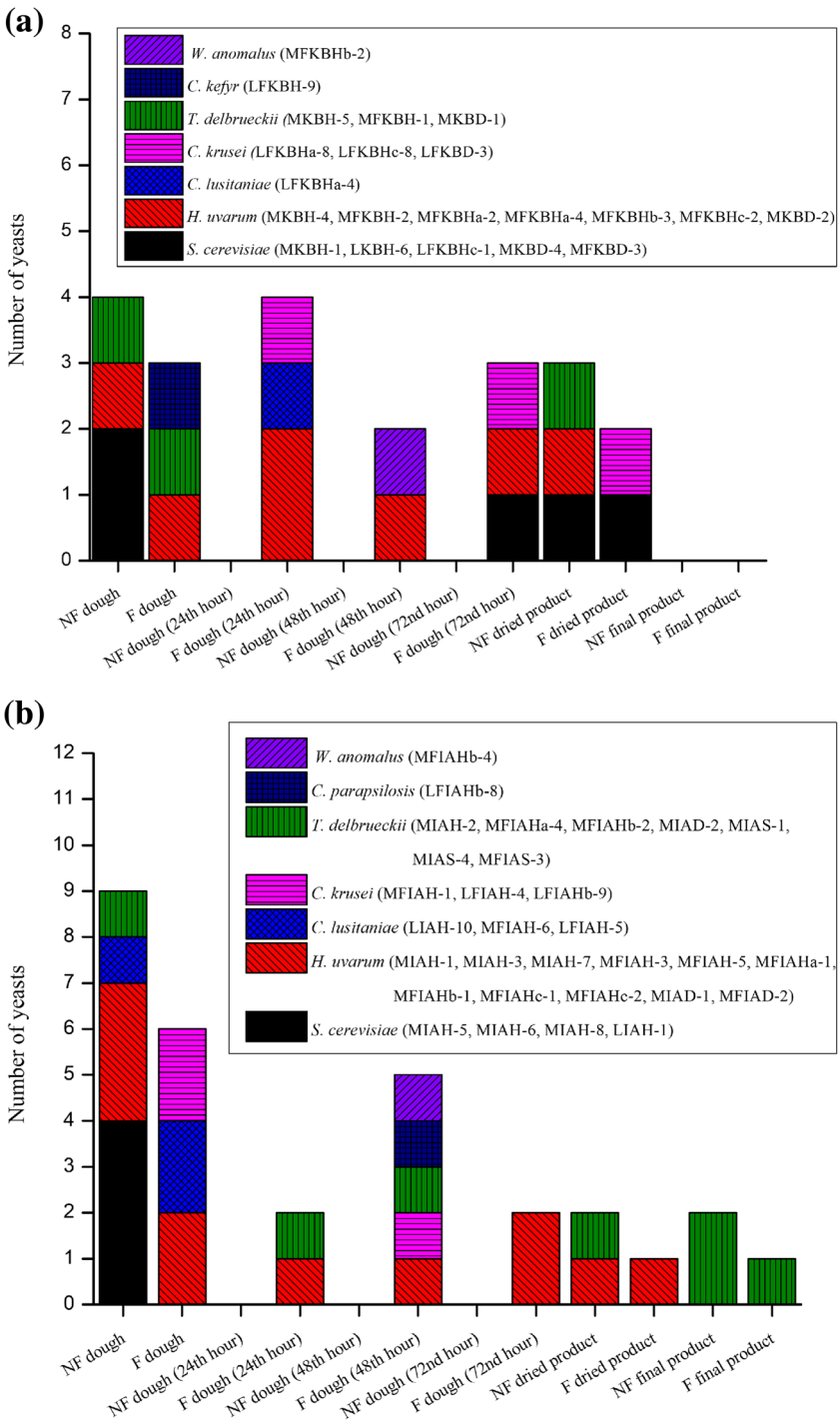
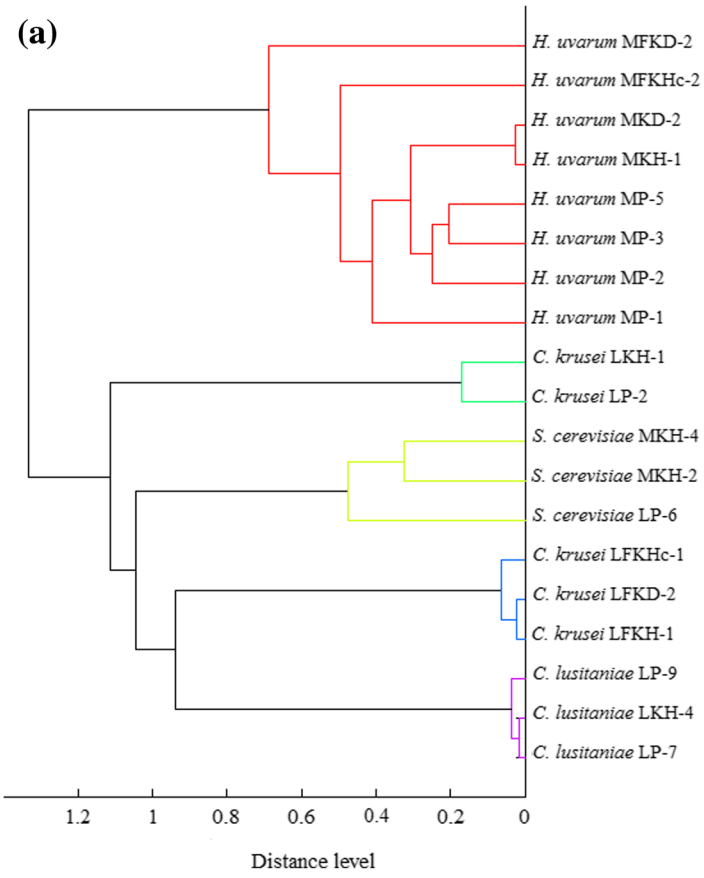


Fig. 3 Identified yeast species from different production steps of the CCT produced with buckwheat flour (**a**) and clear flour (**b**) (F, fermented; NF, non-fermented)

tarhana isolates at a distance level of 0.5. The strains of MFAH-2 and MFAHa-1 isolated from the fermented tarhana dough and the 24th hour of dough fermentation, respectively, were very similar to each other. It could be seen from Fig. 2 that *S. cerevisiae* LAH-4, MAD-1, and LFAD-5 were close to each other. *S. cerevisiae* LP-6 and MAH-5 clustered together and they were distinct from other *S. cerevisiae* strains.

As shown in Fig. 5, *C. krusei* LP-2 did not cluster together with strains of *C. krusei* LFKBD-3 and LFKBHa-8 in the dendogram demonstrating strains of cornelian cherry puree and CCT with buckwheat flour. Although *C. lusitaniae* LP-7 and LP-9 strains obtained from puree were close to each other, *C. lusitaniae* LFKBHa-4 was relatively distinct from (0.4) these isolates. All *S. cerevisiae* strains in CCT with buckwheat flour clustered together. The strains of MKBD-4 and MKBH-1 were very close to each other. *H. uvarum* strains of cornelian cherry puree clustered as a distinct branch and they were relatively distinct from other *H. uvarum* strains.



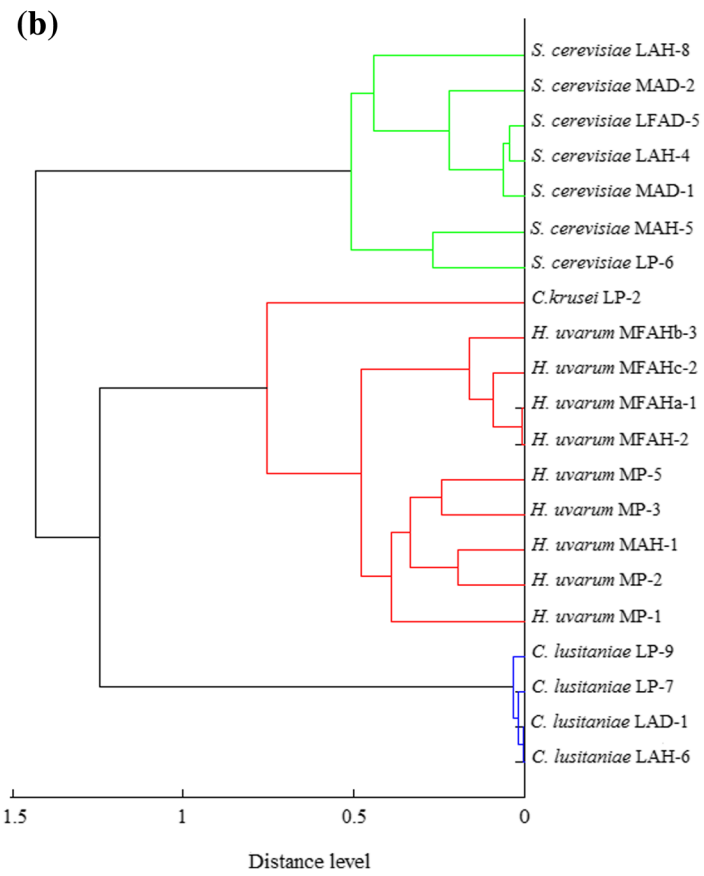


Fig. 4 Cluster analysis of MALDI-TOF/TOF mass spectra of strains belonging to CCT productions performed with wheat flour (**a**) and wholegrain hull-less barley flour (**b**) and their comparison to strains of cornelian cherry puree (F, fermented; P, cornelian cherry puree; K, CCT produced with wheat flour; A, CCT produced with hull-less barley flour; H, dough; a, b ,and c, 24th, 48th, and 72nd hours of fermentation, respectively; D, dried product; S, final product; M and L, specific codes independent from process stages)

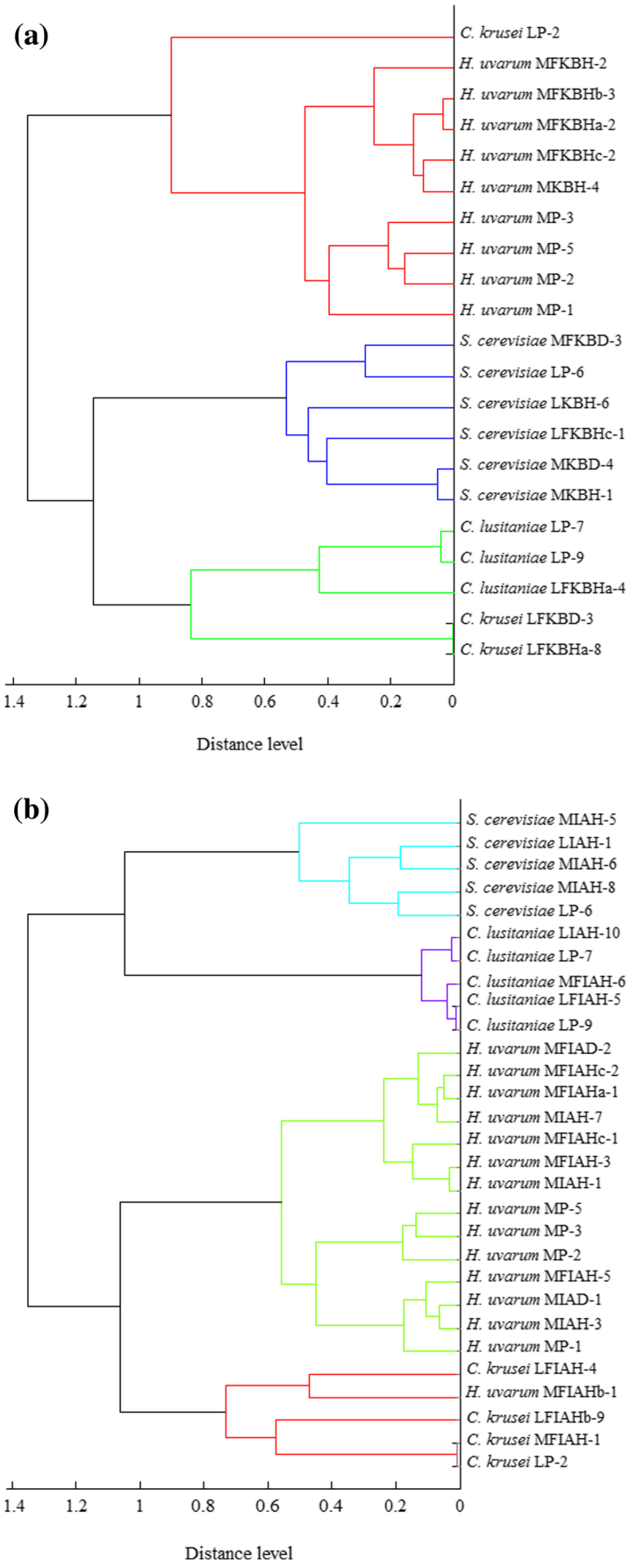


Fig. 5 Cluster analysis of MALDI-TOF/TOF mass spectra of strains belonging to CCT productions performed with buckwheat flour (**a**) and clear flour (**b**) and their comparison to strains of cornelian cherry puree (F, fermented; P, cornelian cherry puree; KB, CCT produced with buckwheat flour; IA, CCT produced with clear flour; H, dough; a, b, and c, 24th, 48th, and 72nd hours of fermentation, respectively; D, dried product; S, final product; M and L, specific codes independent from process stages)

It was demonstrated in Fig. 5 that *C. krusei* strains isolated from cornelian cherry puree (LP-2) and fermented CCT with clear flour (MFIAH-1) were determined to be similar strains. *C. lusitaniae* LP-9 and LFIAH-5 were also very close to each other. It could be observed that some *H. uvarum* strains such as MFIAH-3 and MIAH-1 were close to each other; however, they clustered as a distinct branch from cornelian cherry puree isolates. Strains of *S. cerevisiae* obtained from tarhana dough, particularly *S. cerevisiae* MIAH-5, might be distinct from *S. cerevisiae* LP-6 which was encountered in puree, and could be originated from process equipment.

Discussion

In this study, CCT productions were performed by traditional method and also by fermentation. Microflora of CCT products was investigated for the first time during different production stages. The species of *H. uvarum* (anamorph: *Kloeckera apiculata*), which have been typically isolated from several production stages of CCT products, is one of the apiculate yeasts involved in the spontaneous fermentation of grape fruit together with *S. cerevisiae* (Tristezza et al. 2016). It could be emphasized that yeast microflora of the tarhana products was clearly dominated by the *H. uvarum*. Besides, the only yeast species isolated from the final products of some non-fermented CCT samples and also from majority of fermented CCT products was observed as *T. delbrueckii*. Species of *T. delbrueckii* occur in fruit juices and spontaneously fermented food because of its specific properties including the use of maltose and lactate, producing ester compounds and different fruity aromas in addition to being osmotolerant to 10% NaCl or 5% glucose (De Vuyst et al. 2016; Canonico et al. 2017). So, these could be associated with the isolation of this species from cornelian cherry tarhana products containing approximately 5.5–6% of NaCl. As a remarkable point, the species of *M. pulcherrima* known as one of the yeasts mainly isolated from fruits could not be isolated from cornelian cherry although it was detected in doughs and dried products of some CCT. It was noteworthy that *Cy. fabianii* was not isolated from any production stages of non-fermented or fermented CCT with clear flour, although it was detected in the clear flour. *Cy. fabianii* (previously *Hansenula fabianii*, *Pichia fabianii*, and *Lindnera fabianii*) is a yeast species known as a human pathogen (Joo et al. 2015). In addition to the yeasts, *Bacillus* spp. (11) were identified from CCT products. It was reported that *Bacillus* spp., which are Gram-positive spore forming bacteria, were isolated from biofilms in several environments such as food and beverage industry, and endospores of these bacteria could also contaminate raw materials such as flour (Viedma et al. 2011; Faille et al. 2014).

A limited number of studies were conducted to identify lactic acid bacteria (Sengun et al. 2009; Settanni et al. 2011; Simsek et al. 2017) and yeast microflora (Settanni et al. 2011; Ozel et al. 2015) of traditional tarhana products. In a reported study, several lactic acid bacteria isolates including 12 species from 6 different genera as *Pediococcus*, *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Leuconostoc*, and *Weissella* were identified using a combination of pheno and genotypic methods in tarhana products collected from eight different cities of Turkey (Sengun et al. 2009). In the other study, lactic acid bacteria isolates from tarhana dough fermentation were identified to be *Pediococcus acidilactici*, *Lactobacillus plantarum*, and *Lactobacillus brevis* by a combined genetic approach consisting of 16S/23S rRNA intergenic spacer region (ITS) and partial 16S rRNA gene sequencing (Settanni et al. 2011). The main species for homemade tarhana was found to be *L. plantarum by* Simsek et al. (2017) and it was also reported that microflora of commercial tarhana dough fermentation predominantly composed of *L. brevis* and *Lactobacillus alimentarius*. In the present study, LAB could not be isolated from CCT products and the microflora was mainly composed of yeasts. The major reason of this could be that CCT differed from traditional tarhana and yoghurt is not used in the formulation of CCT products while cornelian cherry is the main ingredient.

In a reported study, the yeast isolates of homemade and commercial traditional tarhana dough were identified to be species of *Pichia kudriavzevii*, *Candida glabrata*, *Candida humilis*, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Kazachstania servazzi*, and *Kazachstania unispora* by using analysis of LSU and ITS-5.8S rDNA sequence (Ozel et al. 2015). In another study, the yeasts from tarhana fermentation were identified by the restriction fragment length polymorphism (RFLP) of the 5.8S ITS rRNA gene to be *Rhodotorula glutinis* and also predominantly *S. cerevisiae* (Settanni et al. 2011). As could be seen from those studies, the yeast microflora of traditional tarhana was reported to be rich in *S. cerevisiae* similarly to our study. However, CCT is a special product that differs from classical tarhana, being mainly composed of cornelian cherry instead of yoghurt. Therefore, its yeast microflora also included *H. uvarum*, *M. pulcherrima*, *T. delbrueckii*, *W. anomalus*, and *Candida* spp. The distinctness in formulation of CCT products might be possible reason to its unique microflora and diverse yeast species.

It is obvious from the results that the fermentation process promoted both yeast growth and diversity of yeast genus and species. Although common species were found in both non-fermented and fermented products, *Wickerhamomyces* spp. and *Pichia* spp. along with *C. parapsilosis*, *W. anomalus*, and *C. kefyr* could be isolated from only fermented CCT.

MALDI-TOF mass spectrometry is a novel high throughput, fast, and cost-effective identification method based on the analysis of whole cell proteins, which offers the possibility of accurately identify to the genus, species, and subspecies levels belong to clinical, enviromental, and foodborne bacteria and yeast isolates (Lv et al. 2016; Quintilla et al. 2018). A MALDI-TOF mass spectrometer is composed of an ion source for solid samples

coupled to mass analyzer that sorts the ions according to their time-of-flight to travel a given distance in a free-field environment (Nacef et al. 2017). There are only a few studies on identifying the microorganisms and particularly yeasts from traditional fermented food products by using MALDI-TOF MS. Some of these studies were about evaluation of lactic acid bacteria and yeast identification results obtained from both proteomic approach using MALDI-TOF MS and genotypic analyzing methods. In a study conducted by Kim et al. (2017), rapid identifying capability of MALDI-TOF MS was confirmed that *Weisella* species isolated from Korean fermented foods and previously identified by 16S rRNA sequencing could be also correctly re-identified. In another study, the same lactic acid bacteria species of traditional French cheese could be identified from both of the analyzing methods, even though two of the 10 isolates were practically identified at genus level by MALDI-TOF MS (Nacef et al. 2017). To identify the bacterial communities of traditional fermentation starters for Chinese rice wine, the MALDI-TOF MS was demonstrated to be a faster and more accurate method because of particularly being useful for the discrimination of closely related species such as *Bacillus* spp. and *L. plantarum* group (Lv et al. 2016). It was reported by Spitaels et al. (2015) that the yeast isolates obtained from traditional beer fermentation were identified through both MALDI-TOF MS and D1/D2 26S rRNA or *ACT*1 gene sequences analysis. On the other hand, there have been also some studies using only genotypic methods to identify microorganisms of several traditional cereal-based fermented foods and beverages (Osorio-Cadavid et al. 2008; Kumar et al. 2010; Sekwati-Monang and Gänzle 2011; Pedersen et al. 2012).

Apart from the reported studies, MALDI-TOF/TOF MS which provides double resolution was used in the present study instead of MALDI-TOF MS (Suckau et al. 2003). Approximately 75% of the isolates could be identified and the microflora of CCT products was investigated for the first time. Dendograms showed that some of the yeast species isolated during production stages were originated from cornelian cherry although source of the others were probably process equipment. The yeast microflora of CCT samples including several species of *Candida*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Saccharomyces*, and *Torulaspora* was determined to be substantially similar to microflora of wine. As it was reported by Capozzi et al. (2015), these yeast species were encountered also in grape and wine environments. The possible reason of this similarity could be the high amount of cornelian cherry fruit in the CCT formulation.

Conclusion

With this study, microflora of cornelian cherry tarhana produced from different raw materials (bread wheat, wholegrain hull-less barley, buckwheat, and clear flours) and methods were demonstrated for the first time. Since cornelian cherry was found to be rich in yeast count, microflora during CCT production was also dominated by yeasts. Common yeast species isolated from all CCT productions were determined to be *H. uvarum*, *S. cerevisiae*, *T. delbrueckii*, *M. pulcherrima*, *C. krusei*, and *C. lusitaniae.* In addition, a few number of lactic acid bacteria could be detected in only raw materials and 11 isolates of *Bacillus* spp. were obtained from production stages of fermented CCT with buckwheat and clear flours. Fermentation induced both yeast growth and diversity. The use of dendograms gave idea about the origins of the strains. This study introduced novel species–identified, endogenic yeasts which could have potential technological characteristics.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A

Informed consent N/A

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