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Identification of lactic acid bacteria and yeasts from traditional sourdoughs and sourdough production by enrichment

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Abstract: The subject of this study was to investigate lactic acid bacteria (LAB) and yeasts microbiota of traditional sourdough (n = 36) and to indicate characteristics of enriched sourdough that is produced from combinations of isolates. A total of 60 LAB and 40 yeasts were identified from sourdough by matrix-assisted laser desorption ionisation--time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The dominant LAB microflora was *Lactobacillus brevis* (43.33%), *Pediococcus acidilactici* (21.67%) and *Lactobacillus plantarum* (18.33%). The dominant yeasts microflora was *Saccharomyces cerevisiae* (27.5%), *Pichia kudriavzevii* (25.0%) and *Kluyveromyces marxianus* (12.5%). The sourdough prepared with the combination of *L. brevis, Leuconostoc mesenteroides, P. acidilactici* and *S. cerevisiae, K. marxianus* showed the best physicochemical and microbiological properties while that with *L. plantarum, L. brevis* and *P. kudriavzevii, Wickerhamomyces anumalus* was the poorest. LAB and yeasts in the sourdoughs were ranged from 6.58 log CFU g^{-1} to 9.12 log CFU g^{-1} and from 6.12 log CFU g^{-1} to 7.88 log CFU g^{-1}, respectively. Various chemical parameters such as pH, total titratable acidity (TTA), ethanol, and sourdough volume were differ depending on the type of microbial species. TTA was more pronounced in the sourdoughs produced with homofermentative LAB. Yeasts and LAB were dominated during continuous enriching of sourdough, supporting an important role during fermentation.

Keywords: traditional sourdough; lactic acid bacteria; yeasts; microbiota; enrichment

The production of bread can be traced back to ancient times. This tradition has passed through some stages in the later ages and has reached the present day and is still being used as a fermentation practice. The sourdough is a mixture of flour and water that is fermented with lactic acid bacteria (LAB). Sourdough fermentation is generally evaluated by the measurement of parameters such as pH, acidity and microbiota (Gul et al. 2005; Minervini et al. 2012). Taxonomic microbial identifications can be performed by phenotypic characterisation and matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry (MALDI-TOF MS) analysis (Kanak and Yılmaz 2018). Microorganisms can be rapidly identified with MALDI--TOF MS technique, which is based on the principle of ionising specific protein profile of microbial cells. In this technique, protein fingerprints of microorganisms are compared with the references in the system's database (Xu 2017). Bread quality mainly depends on the sourdough quality and regionally involves different types of LAB and yeasts. There are various studies on the flora of Turkish sourdoughs from different regions of Turkey. Some researchers have studied on the isolation of LAB and yeasts from sourdough obtained from Isparta (Gul et al. 2005), Kütahya, Ankara and Adana (Yagmur et al. 2016), Ankara, Mersin and

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Antalya (Boyaci-Gunduz and Erten 2020), Uşak (Simsek et al. 2006) and Trabzon (Dertli et al. 2016) in Turkey. They observed different microbiota with different quality characteristics of sourdough that is used in the production of sourdough bread. However, researches on the identification of sourdough microbiota from Gaziantep, Mardin and Konya have not been conducted. These regions have a long sourdough bread production history and different sourdough bread characteristics. This study focuses on the isolation and identification of LAB and yeasts from traditional sourdoughs obtained from Gaziantep, Mardin and Konya provinces by morphological, biochemical and MALDI-TOF MS analysis. The characteristics of sourdoughs produced with enriching from a combination of isolated LAB and yeasts were also studied. This study assists in determining the appropriate combination of isolates in sourdough production and choosing the beneficial LAB and yeast to obtain high-quality products.

MATERIAL AND METHODS

Traditional (homemade) sourdough samples (n = 36) were obtained from Gaziantep, Mardin and Konya provinces in Turkey. Samples were carried under chill conditions and stored in a refrigerator until use. All sourdoughs were obtained in duplicate and analyses were performed with duplicate samples.

Isolation and identification of LAB and yeasts. Sourdough sample (25 g) was weighed (Model CC062D-10ABAAGA; Avery Berkel, United Kingdom) and added into 225 mL of sterile peptone water (0.1%) in the Waring blender (8011ES; Waring Commercial, USA) and homogenised by blending for 1 min. LAB and yeasts were isolated and purified (ES 500; NUVE, Turkey), as indicated by Aplevicz et al. (2014) and Erkmen (2015). The pure cultures were stored in 20% (v/v) glycerol and stored at –20 °C until use in the identification tests (Eppendorf tube; Isolab, Turkey). LAB and yeasts were firstly tested for morphological identification tests as indicated by Abegaz (2007), Aplevicz et al. (2014) and Erkmen (2015, 2021). After identification of LAB and yeasts at the genus level, they were identified at species level by MALDI-TOF MS analysis (Microflex LT; Bruker Daltonics GmbH, Germany).

Preparation of sourdough. Each LAB and yeast species identified from traditional sourdough were preparated separately in 100 mL de Man, Rogosa and Sharpe (MRS) broth and potato dextrose (PD) broth, respectively. The cultures were centrifuged (6 000 × g for 15 min, Andreas Hettich, Germany) separately, washed twice with physiological saline solution (PSS) and centrifuged again. The pellets were resuspended in PSS to obtain stock culture which is used in the preparation of mother culture. The mother culture for the preparation of bulk sourdough culture (sourdough) was obtained by mixing an equal amount of each microbial stock culture (MSC). While determining the combinations of LAB and yeasts to be used in sourdough production, technological properties of microorganisms were taken into consideration as indicated by Gul et al. (2005), Valmorri et al. (2010), Yagmur et al. (2016), Erkmen (2015) and Arici et al. (2018). In this research, six combinations of LAB and yeasts were made to produce sourdoughs (Table 1). The production flowchart of the sourdough culture is given in Figure 1. The sour-

Table 1. Combinations of LAB and yeasts used in the sourdough production

Sourdough type	Sourdough culture combinations
SD1	*L. brevis* + *L. plantarum* + *L. paraplantarum* + *P. kudriavzevii* + *W. anomalus*
SD2	*L. plantarum* + *P. acidilactici* + *E. faecalis* + *K. unispora* + *C. tropicalis* + *C. glabrata*
SD3	*L. brevis* + *L. plantarum* + *L. paraplantarum* + *L. pentosus* + *L. mesenteroides* subsp. *cremoris* + + *S. cerevisiae* + *K. unispora* + *W. anomalus*
SD4	*L. brevis* + *L. mesenteroides* subsp. *cremoris* + *S. cerevisiae* + *K. unispora*
SD5	*P. acidilactici* + *L. plantarum* + *L. mesenteroides* subsp. *mesenteroides* + *K. marxianus*
SD6	*L. brevis* + *L. mesenteroides* subsp. *mesenteroides* + *E. hirae* + *L. plantarum* + + *P. acidilactici* + *L. pentosus* + *S. cerevisiae* + *K. marxianus*

LAB – lactic acid bacteria; SD – sourdough; *L. brevis* – *Lactobacillus brevis*; *L. plantarum* – *Lactobacillus plantarum*; *L. paraplantarum* – *Lactobacillus paraplantarum*; *P. kudriavzevii* – *Pichia kudriavzevii*; *W. anomalus* – *Wickerhamomyces anomalus*; *P. acidilactici* – *Pediococcus acidilactici*; *E. faecalis* – *Enterococcus faecalis*; *K. unispora* – *Kazachstania unispora*; *C. tropicalis* – *Candida tropicalis*; *C. glabrata* – *Candida glabrata*; *L. pentosus* – *Lactobacillus pentosus*; *L. mesenteroides* – *Leuconostoc mesenteroides*; *S. cerevisiae* – *Saccharomyces cerevisiae*; *K. marxianus* – *Kluyveromyces marxianus*; *E. hirae* – *Enterococcus hirae*

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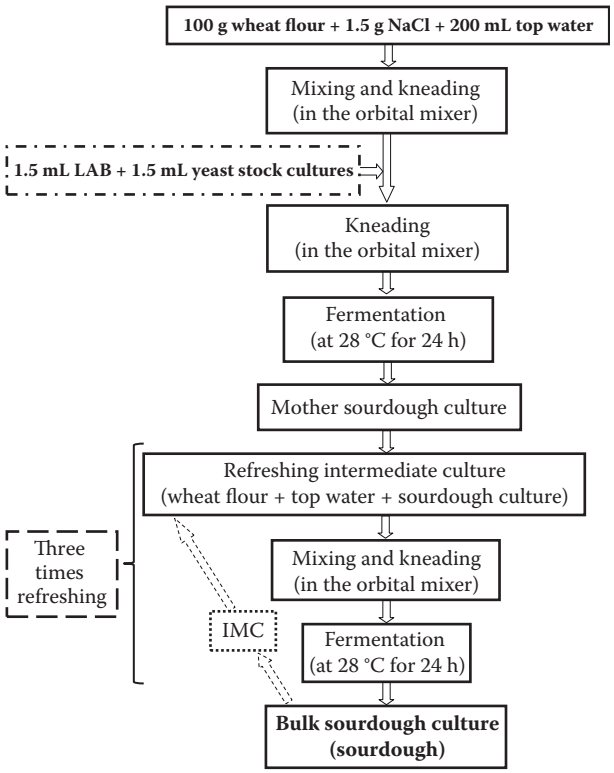

Figure 1. The sourdough production flowchart

IMC – intermediate culture; LAB – lactic acid bacteria

dough was prepared from wheat flour (WF) (Golia type wheat). WF was supplied from Özmen flour industry and Trade Inc. (Gaziantep, Turkey). This first sourdough culture MSC was used in the preparation of sourdough by three times enrichment (refreshing) through intermediate culture (IMC) with the following ingredients base:

i) Enrichment: 100 g WF + 205 mL water + 60 g MSC was mixed in the mixer and left to fermentation at 28 °C for 24 h that was used as IMC-1.

ii) Enrichment: 135 g WF + 200 mL water + 65 g IMC-1 was mixed in the mixer and left to fermentation at 28 °C for 24 h that was used as IMC-2.

iii) Enrichment: 135 g WF + 200 mL water + 65 g IMC-2 was mixed in the mixer and left to fermentation at 28 °C for 24 h. Sourdough was used in the determination of sourdough physicochemical and microbiological properties.

Initial numbers of LAB and yeast in the mixed ingredients in the first enrichment mixture before incubation were ranged from 5.58 colony-forming unit (CFU) g^{-1} to 5.94 CFU g^{-1} and 4.64 CFU g^{-1} to 4.88 CFU g^{-1}, respectively. Initial numbers of mesophilic aerobic bacteria (MAB) in the mixed ingredients in the first enrichment mixture before incubation were ranged from 5.49 CFU g^{-1} to 5.62 CFU g^{-1}.

Determination of sourdough properties. Samples (25 g) were taken from sourdough. In the analysis, 25 g of sourdough was added into a sterile Warring blender (8011ES; Waring Commercial, USA) containing 225 mL of sterile distilled water and blended for 1 min. LAB, mesophilic aerobic count (MAC) and yeasts counts were performed according to the methods indicated by Erkmen (2015). The results were indicated as a CFU g^{-1} of sourdough. TTA (% lactic acid) and pH analysis were performed as indicated by Paramithiotis et al. (2006).

Sourdough volume. The sourdough sample (125 g) was placed into a sterile 1.0 L graduated cylinder (Isolab, Turkey) and was left to incubate for 4 h in the incubator at 28 °C (ES 500; NUVE, Turkey). The initial volume (V_i in mL) and the final volume (V_f in mL after 4 h) of the dough samples were recorded. The change in the volume of the dough sample was calculated in % using the following formula:

$$Volume\ (\%) = [(V_f - V_i)/V_i] \times 100 \quad (1)$$

where: V_f – final volume (mL); V_i – initial volume (mL).

Ethanol content. Ethanol in the sourdough was determined using high-performance liquid chromatography (HPLC) equipped with a refractive index detector (RID-10A; Shimadzu, Japan) and a Shodex SH 1011 column (7 µm, 8 × 300 mm; Shimadzu, USA) at 35 °C according to Paramithiotis et al. (2006). Ethanol amount was calculated as mL kg^{-1} using a standard curve.

Statistical analysis. Sourdough production was repeated three times in a separate day, each repeat was run in parallel, and a parallel sample was used in each analysis. The results of all analyses were evaluated by IBM SPSS Statistic 22 program. One-way analysis of variance and ANOVA test were used in the statistical analysis. Between differences, $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

To bacilli belonged 74.51% of LAB, and others (25.49%) are identified as cocci. LAB identified from sourdoughs were given in Tables 2 and 3. The most LAB species diversity (8 species) was determined from Gaziantep sourdoughs, while the less LAB species (5 species) were detected from the Mardin sourdoughs. Eleven types of LAB species were isolated from 36 sour-

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Table 2. MALDI-TOF MS analysis results of LAB (n = 60) from traditional sourdoughs (n = 36) obtained from three cities in Turkey

LAB	(%)
LAB from Konya (*n* = 17; 28.33%)	
Pediococcus acidilactici	41.18
Lactobacillus brevis	23.53
Lactobacillus plantarum	17.64
Lactobacillus pentosus	5.88
Weissella confusa	5.88
Enterococcus hirae	5.88
LAB from Gaziantep (*n* = 19; 31.67%)	
Lactobacillus brevis	42.10
Lactobacillus plantarum	21.05
Pediococcus acidilactici	10.52
Lactobacillus paraplantarum	5.26
Lactobacillus pentosus	5.26
Enterococcus faecalis	5.26
Lactobacillus paralimentarius	5.26
Enterococcus hirae	5.26
LAB from Mardin (*n* = 24; 40.0%)	
Lactobacillus brevis	58.33
Lactobacillus plantarum	16.66
Pediococcus acidilactici	16.66
Leuconostoc mesenteroides subsp. *mesenteroides*	4.16
Leuconostoc mesenteroides subsp. *cremoris*	4.16

LAB – lactic acid bacteria; MALDI-TOF MS – matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry

Table 3. MALDI-TOF MS analysis results yeasts (n = 40) from traditional sourdoughs (n = 36) obtained from three cities in Turkey

Yeasts	(%)
Yeasts from Konya (*n* = 18; 45.0%)	
Pichia kudriavzevii	38.88
Kluyveromyces marxianus	22.22
Geotrichum candidum	16.66
Kazachstania unispora	11.11
Galactomyces candidum	5.55
Candida kefyr	5.55
Yeasts from Gaziantep (*n* = 13; 32.50%)	
Pichia kudriavzevii	23.07
Saccharomyces cerevisiae	23.07
Wickerhamomyces anomalus	15.38
Kazachstania humilis	15.38
Candida glabrata	15.38
Candida tropicalis	7.69
Yeasts from Mardin (*n* = 9; 22.50%)	
Saccharomyces cerevisiae	88.88
Kluyveromyces marxianus	11.11

MALDI-TOF MS – matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry

doughs. The more yeast species (6 species) were determined from both, Gaziantep and Konya sourdoughs, while the less species (2 species) were detected from Mardin sourdoughs. The data obtained emphasise that the diversity of sourdough LAB and yeasts changes regionally. There are differences in the technological characteristics of LAB species. Heterofermentative LAB are more frequently (73.33%) identified than homofermentative. While only *Pediococcus acidilactici* shows oxidative properties, the remaining species are determined to be fermentative. It is important for LAB to produce gas and acid in the presence of sugars. It was determined that all LAB produce acid from glucose while only 54.90% of them produce gas. While 52.94, 3.92, and 3.92% of LAB produced gas and acid from maltose, lactose and sucrose, respectively, the rest of them produced acid only. Important features of isolated LAB well grow in the presence of salt and production of acid and gas from sugars at 28 °C. While all the LAB grows well at 28 °C, only *P. acidilactici* cannot grow at 15 °C and only *P. acidilactici* and *Enterococcus* spp. grow at 45 °C. As a result of the research, all LAB can achieve good growth at the 1.5% salt concentration to be used in the sourdough industry.

There were differences in the technological characteristics of yeast species (Table 3). In the environment where glucose is present, all yeasts grow, and 55.56% of them produced gas. While 75% of the yeasts grow in the environment containing sucrose, only 59.26% of them produced gas. In the presence of lactose and maltose, 8.33% and 72.21% of yeasts, respectively, grow without gas production. All yeasts grow at 25 °C and 28 °C. It was determined that both LAB and yeasts were grown well at 28 °C used as fermentation temperature in the sourdough industry.

The pH, TTA, sourdough volumes and ethanol values of sourdoughs are given in Table 4. During the fermentation of sourdough, the pH values varied between 3.43 and 4.15. The change of pH in sourdough samples mostly depends on the number of species and their homofermentative characteristics. Low pH ($P < 0.05$) was determined especially in sourdough SD6, SD3

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Table 4. Physicochemical characteristics of sourdoughs

Sourdough	pH	Titratable acidity (%)	Sourdough volume (%)	Ethanol (mL kg^{-1})
Control	5.62 ± 0.01^{a}	3.34 ± 0.08^{a}	140.87 ± 12.4^{a}	208.42 ± 10.5^{a}
SD1	3.62 ± 0.08^{b}	3.36 ± 0.06^{b}	156.56 ± 10.1^{b}	159.87 ± 9.8^{b}
SD2	3.43 ± 0.03^{c}	3.90 ± 0.06^{c}	180.87 ± 10.1^{c}	132.56 ± 10.5^{c}
SD3	3.59 ± 0.08^{c}	2.77 ± 0.04^{c}	165.10 ± 10.3^{c}	114.54 ± 10.5^{c}
SD4	4.15 ± 0.01^{d}	3.25 ± 0.10^{b}	153.54 ± 10.3^{b}	162.37 ± 10.4^{b}
SD5	3.60 ± 0.15^{b}	3.67 ± 0.04^{b}	162.60 ± 10.3^{b}	148.78 ± 10.4^{b}
SD6	3.45 ± 0.03^{c}	3.95 ± 0.02^{d}	189.12 ± 10.3^{d}	124.15 ± 11.2^{c}

$^{a–d}$Same letter indicates no significant difference ($P > 0.05$), different letters indicate significant difference ($P < 0.05$); SD – sourdough

and SD2 with the use of more homofermentative LAB. Similar results ($P < 0.05$) were also detected for TTA for SD6, SD3 and SD2 according to the changes in pH values. TTA values of sourdough varied between 3.25 and 3.95. Control dough volume was less than sourdough volumes ($P < 0.05$). A little more sourdough volume was detected in the SD6, SD3 and SD2 ($P < 0.05$) than others due to the contents of more heterofermentative LAB species and a high number of initial LAB types. Heterofermentative LAB may produced more gas together with yeasts which resulted in a high-volume content of sourdough. On the other hand, more ethanol production ($P < 0.05$) occurred in the control dough (Table 4). This may be due to the high number of LAB in sourdoughs competing with yeasts for substrate and resulted in restricting the substrates where yeasts can be used to produce ethanol. The low acid environment may also reduced ethanol production, as appeared in SD6, SD3 and SD2 ($P < 0.05$) than the others. In this research, ethanol levels were lower (114.54–159.87 mg kg^{-1}) than those reported by Yagmur et al. (2016); they indicated higher ethanol levels 7.72–14.79 g kg^{-1} in fermentations of LAB and yeasts while they were used as single or two species in the fermentation.

The number of LAB varied between 6.58 log CFU g^{-1} and 9.12 log CFU g^{-1}. The use of more types of LAB in sourdough production resulted in a higher number of LAB. This situation was found especially for the SD6, SD3 and SD2 sourdoughs ($P < 0.05$). Yeasts in sourdough ranged from 5.89 log CFU g^{-1} to 7.88 log CFU g^{-1} (Table 5). More types of yeasts were used in the production of SD2 and SD3; therefore, higher numbers of yeasts were detected in these sourdoughs than the others ($P < 0.05$). Yeasts and LAB grow together; there is no identifiable inhibition among these microorganisms except competition for a substrate. Another important result of the study is that the isolated LAB and yeasts do not have negative effects on each other in sourdough fermentation. LAB is stimulated by CO_2 production by yeasts. Yeasts produce growth factors stimulatory to LAB in the sourdough environment (Paramithiotis et al. 2006). As a result of sourdough fermentation, the number of MAB varied between 4.10 log CFU g^{-1} and 5.16 log CFU g^{-1} (Table 5). MAB number was lower in sourdoughs with higher numbers of LAB used in sourdough fermentation. This situation appears between SD4 and others ($P < 0.05$). Due to the increase in the number of LAB in sourdough, the variability of MAB was restricted. MAB count was higher in the control dough than sourdoughs ($P < 0.05$). The lactic acid production negatively affected the microbiota of sourdoughs.

Robert et al. (2009) indicated that heterofermentative LAB represent more than 76% of the total isolates; the main species isolated were *Lactobacillus planta-*

Table 5. Microbiological characteristics of sourdoughs [(log CFU) g^{-1}]

Sourdough	LAB	MAB	Yeasts
Control	2.98 ± 0.09^{a}	5.88 ± 0.03^{a}	7.97 ± 0.02^{a}
SD1	6.86 ± 0.12^{b}	4.68 ± 0.09^{b}	5.89 ± 0.06^{b}
SD2	8.64 ± 0.09^{c}	4.14 ± 0.02^{c}	7.88 ± 0.06^{c}
SD3	8.62 ± 0.09^{c}	4.26 ± 0.08^{c}	7.01 ± 0.05^{c}
SD4	6.58 ± 0.03^{b}	5.16 ± 0.12^{d}	6.80 ± 0.06^{d}
SD5	7.18 ± 0.08^{b}	4.61 ± 0.09^{b}	6.12 ± 0.03^{b}
SD6	9.12 ± 0.01^{c}	4.10 ± 0.12^{c}	6.01 ± 0.03^{b}

$^{a–d}$Same letter indicates no significant difference ($P > 0.05$), different letters indicate significant difference ($P < 0.05$); CFU – colony-forming unit; LAB – lactic acid bacteria; MAB – mesophilic aerobic bacteria; SD – sourdough

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rum and *Pediococcus pentosaceus.* Similar heterofermentative results (73.33%) were found in this study, while *Lactobacillus brevis* (43.33%), *Pediococcus acidilacticis* (21.67%) and *L. plantarum* (18.33%) were the main isolates. Valmorri et al. (2010) indicated that 85% of the yeast isolates were *Saccharomyces cerevisiae*. In this study, *S. cerevisiae* was isolated by 27.5% among all yeast isolates, and *L. brevis* was isolated by 43.33% among all LAB isolates. On the other hand, this yeast was not isolated from Konya sourdoughs. When the results of sourdough obtained from different regions were compared, there are high variations in the LAB and yeasts among sourdough microbiota. Low numbers of LAB and yeasts species were isolated from Mardin compared to Gaziantep, while both cities are located in the Southeast region of Anatolia in Turkey. The microbial ecology of sourdough results from geography and traditional practices (De Vuyst and Vancanneyt 2007). Therefore, the distribution of the microbiota of LAB is highly variable from one sourdough ecosystem to another; many sourdoughs have associations of heterofermentative and homofermentative LAB. Homofermentative LAB produce a higher amount of lactic acid. Heterofermentative ones produce ethanol, acetic acid and CO_2 besides lactic acid from sugars to provide aroma and flavour compounds (Erkmen and Bozoglu 2016). Homofermentative LAB influenced the pH reduction by a significantly higher ($P < 0.05$) amount of lactic acid production compared with heterofermentative in sourdough fermentation. There was significant ($P < 0.05$) variations in the acidity among sourdough types (Table 4). In this research, four successive enrichment sourdough fermentation have been conducting by fresh substrate at every case. Different species of LAB and yeasts can be dominated during this long-time continuous enrichment of sourdoughs, supporting an important role of LAB and yeasts. Microbial persist in the microbiota is ascribed to a competitive metabolism and adaptation during fermentation. The use of competitive microbial species might help to develop new, stable and controlled sourdough starter cultures for sourdough fermentation processes.

CONCLUSION

In recent years, the high consumption of bread has led to more studies on the bread production steps. Sourdough that isolated traditional sourdough is mainly composed of heterofermentative species. *L. brevis*, *P. acidilactici* and *L. plantarum* were dominated flora in the sourdough microbiota. The most common yeasts were *S. cerevisiae, Pichia kudriavzevii* and *Kluyveromyces marxianus* in the sourdough microbiota. This study assists in determining the appropriate combination in sourdough production from isolates for choosing the appropriate LAB and yeasts to obtain high-quality bakery products. The bread quality varies depending on the regional sourdough type. The pH results of homofermentative LAB cause a higher pH decrease. When compared with the control dough, the characteristics of sourdough made with the combination of LAB and yeasts were better. The use of competitive microbial species might help to develop new, stable and controlled sourdough starter cultures for sourdough fermentation processes. LAB and yeasts were dominated during continuous enrichment of sourdoughs supporting microbial species important role during fermentation.

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