World Applied Sciences Journal 24 (7): 900-907, 2013
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2013.24.07.13248

Production of Cheese Flavor and Antifungal Substances by *Lactobacillus plantarum and Lactococcus lactis subsp diacetylactis* and Their Application in Bakery Products

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Submitted: Jul 20, 2013; **Accepted:** Aug 29, 2013; **Published:** Aug 29, 2013

Abstract: *Lactobacillus plantarum* and *Lactococcus lactis subsp diacetylactis* were chosen to produce flavored bakery products (for example, pies) on pilot plant scale. In this study, flavors and antimicrobial substances produced by *Lb. plantarum* and *Lc. diacetylactis* were applied for improving the quality & safety of bakery products (i.e. Pies). Aroma volatile constituents, chemical composition, baking quality and freshness and sensory characteristics of pies were determined. Also, strains were evaluated for their antifungal activity against molds. Sensory evaluation showed that there were no significant differences in mouth feel, texture, break &shred, crust color and symmetry shape scores between control pie and all different pie formulas. Taste and palatability of produced pie were improvement for formulas (*Lb. plantarum* and *Lc diacetylactis*) compared with control. The presence of lactic acid bacteria (LAB) metabolites in dough of bakery products increases the shelf life of the products due to the inhibitory effect of organic acids on spoilage molds. In addition, it has been found that when sour dough LAB is cultivated they produce antifungal substances, such as organic acids (in particular, lactic acid and acetic acid), carbon dioxide, ethanol and hydrogen peroxide. From the above results it could be concluded that LAB improved the quality and safety of bakery by production of good flavor and natural antifungal substances could inhibit molds growth. This means that these metabolites could be utilized as natural flavors and preservatives in foods.

Key words: Antifungal activity · Bakery products · Flavor · Lactobacillus plantarum · Lactococcus diacetylactis

INTRODUCTION

Flavor is one of the most valued sensory attributes in bakery- volatile and non-volatile compounds produced during the fermentation of dough contribute to bread flavors. Some studies reports show that the fermentation of dough with Lactic acid bacteria (LAB) can enhance the aroma and flavor [1, 2]. The growth of fungi is responsible for the formation of the off-flavors and the production of mycotoxins; adding LAB to dough can prevent the growth of fungi and enhance the flavor of bread. The produced compounds play an important role for any technological application to enhance the flavor, such as diacetyl which gives a buttery flavor [3]. The combination of bacteria and yeast creates complex aromatic flavors using acids, alcohols, ketones and esters that react with the amino acids in flour during baking to produce more than that 200 different flavor compounds. These flavor compounds contribute to the subtle and distinctive taste and aroma of fresh baked bread. Because the consistency and quality of prepared sour starter cultures has improved markedly over the last five years, volume bakers should be poised to take advantage of the growing trend towards specialty flavored breads [4]. There are two main types of

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lactic acid bacteria: homofermentative and heterofermentative. Homofermentative bacteria, such as *Lb. plantarum* produce lactic acid under anaerobic conditions from maltose or glucose in the dough. These bacteria also produce an elastic crumb in bread. Because homofermentative bacteria do not produce carbon dioxide, yeast must be added to ensure leavening of the dough. On the other hand, heterofermentative lactobacilli, such as *Lb. brevis or Lb. sanfrancisco,* produce a variety of organic compounds, including lactic acid, ethanol and carbon dioxide gas. And, higher production throughput does not always have to mean lower quality taste. "For those bakeries using no-time dough, adding powdered fermentation flavours is a natural way to enhance product appeal and gain the effects of a long-time natural fermentation process". Sprays dried or drum dried fermentation flavors can be added to multigrain, whole wheat or artisan breads to give them a more rounded and complex flavor. Moreover, Lavermicocca *et al.* [5] working with *Lb. plantarum* 21B, isolated from sourdough and identified phenyllactic and 4-hydroxyphenyllactic acids as active compounds with antifungal activity produced by this strain. Mold spoilage of bakery products is a serious economic concern. Losses due to Mold spoilage vary between 1 and 5% of products depending on season, type of product and method of processing. In addition to the economic losses another concern is the possibility that mycotoxins may be produced. LAB have a long history in preserving foods from spoilage microorganisms - they are commonly used in food fermentation, may produce several metabolites with beneficial health effects and, thus, are generally recognized as safe (GRAS). LAB are well known for their antifungal activity, which is related to the production of a variety of compounds including acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide, phenyllactic acid, bacteriocins and cycle peptides [6]. Several species or subspecies such as *Lc. lactis* subsp. *lactis, Lc. lactis* subsp. cremoris, *Lc. lactis* subsp. *diacetylactis, Lb.acidopi1ilus, Lb. plantarum* and *Lb. curvatus* are able to synthesize peptides or antimicrobial proteins known as bacteriocins, whose activity is only directed against eloselytaxonomically-related bacteria. Numerous studies have reported that these molecules are inactive against Gram-negative bacteria and eucaryotic microorganisms such as yeasts or moulds [7]. The aim of this study is to use *Lb. plantarum* and *Lc. lactis subsp diacetylactis* to produce cheese flavor by fermentation in raw milk and use it to manufacture bakery products (For example : Pies) and evaluation of their quality and safety.

MATERIALS AND METHODS

Materials: Wheat flour (72% extraction) was purchased from the North Cairo Flour Mills Company, Egypt. Sugar, shortening, active dry yeast, salt and starters, *Lactococcus lactis* subsp *diacetylactis* and *Lactobacillus plantarum.*

Preparation and Evaluation of Pie: Pies were made in Technological Development for Food Industries Unit (Food Industries & Nutrition-National Research Center) on Pilot Plant Scale. Pie samples were made with different formulas as shown in Table 1. The dough was left to ferment (1h/30ºC/85% relative humidity), then divided to pieces (100g). The pieces were arranged on trays and were left to ferment for about 30 min at the same temperature and relative humidity. The pieces of fermented dough were flattened to be about 10 cm in diameter. The flattened loaves were proofed at 30-35ºC and 85% relative humidity for 15 min and then were baked at 230ºC for 10 min. The pies were allowed to cool on racks for about 1 hr before evaluation. Pies were evaluated organoleptically by 15 trained panelists according to Kulp *et al.* [8]. The tested characteristics were; taste (20), aroma (20), mouth feel (10), crumb texture (15), crumb colour (10), crust color (10), break & shred (10) and symmetry shape (5).

Analytical Methods: Moisture, protein, fat, crude fiber and ash of wheat flour and different pies were determined according to A.O.A.C. [9]. Carbohydrates were calculated by differences. Caloric value was calculated according to the following equation:

$$E = 4 (\text{Protein \%} + \text{Carbohydrate \%}) + 9 (\text{Fat \%}).$$

Color: Changes in Hunter color parameter (L, a &b) of wheat flour and different cakes were followed up using Tristimulus Color Analyzer (Hunter, Lab Scan XE, Reston, Virginia) with standard white tile.

Pie Volume and Weight: The volume of pie was measured by rapeseed displacement. Both weight and volume were determined according to the method described by Kulp *et al.* [8]:

$$\text{Specific volume} = \text{Volume/Weight}$$

Freshness of Pies: Pies freshness was tested after wrapping using polyethylene bags and storing at room temperature (0, 3 and 7 days) using Alkaline Water

Table 1: Composition of formulas used in manufacture of pie.

	Ingredients(g)						
Formulas	Wheat flour	Fat	Yeast	Salt	Sucrose	*Streptococcus diacetilactis*	*Lactobacillus plantarum*
1.Basic formula (control)	100	25	2	1.5	10	-	-
2. Pie with *Lactococcus*	95	20	2	1.5	10	5	-
3. Pie with *Lactobacillus*	95	20	2	1.5	10	-	5

Retention Capacity test (AWRC) according to the method of Yamazaki [10], as modified by Kitterman and Rubenthaler [11].

Flavor Production: Sterilized milk was supplemented with phenylalanine and inoculated with 10% of the inoculum of LAB strains separately and incubated at 35°C (*Lb. plantarum*) or 30°C (*Lac. lactis subsp diacetylactis*) for a period of time sufficient for the bacteria to produce a satisfactory range of flavor compounds and acids in the mixture, virtually free of off-flavors. A fermented dairy product contains a mixture of aromatic compounds and acids. Fermented dairy product obtained was added to a bakery recipe to impart an enhanced flavor to the resulting bakery product, namely a slight acid and a cheese-like flavour. Finally the flavored media was freeze-dried and kept at room temperature. From the aforementioned results [12], *Lactobacillus plantarum* and *Lactococcus diacetylactis* were chosen to produce flavour in food products.

Isolation of Headspace Volatiles: The volatiles in the headspace of each sample under investigation were isolated by using a dynamic headspace system. The samples were purged for 1h with nitrogen gas (grade of N2<99.99) at a flow rate 100 ml/min. the headspace volatiles were swept into cold traps containing diethyl ether and pentane (1:1, v/v) and held at -10°C. The solvents containing the volatiles were dried over anhydrous sodium sulfate for 1 h. the volatiles were obtained by evaporation of the solvents under reduced pressure [13].

Gas Chromatographic (GC) Analysis: GC analysis was performed by using Hewlett-Packard model 5890 equipped with a flame ionization detector (FID). A fused silica capillary column DB-5 (60mx0.32 mm id,) was used. The oven temperature was maintained initially at 50°C for 5 min and then programmed from 50 to 250°C at a rate of 4°C/min. Helium was used as the carrier gas, at flow rate of 1.1 ml/min. The injector and detector temperatures were 220 and 250°C, respectively. The retention indices (Kovats index) of the separated volatile components were calculated using hydrocarbons (C8-C22, Aldrich Co.) as references.

Gas Chromatographic-Mass Spectrometric (GC-MS) Analysis: The analysis was carried out by using a coupled gas chromatography Hewlett-Packard model (5890)/ mass spectrometry Hewlett-Packard MS (5970). The ionization voltage was 70 eV, mass range m/z 39-400 a.m.u. The GC condition was carried out as mentioned above. The isolated peaks were identified by matching with data from the library of mass spectra (National Institute of Standard and Technology) and compared with those of authentic compounds and published data [14]. The quantitative determination was carried out based on peak area integration.

Assay of Antifungal Activity: The antifungal activities of *Lb. plantarum and Lc. lactis subsp diacetylactis* were tested against *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium sp.* by agar well diffusion method outlined by Batish *et al.* [15]. Mold spore suspension and the cell free supernatant were prepared by the procedure outlined by Batish *et al.* [15].

Statistical Analysis: The obtained results were evaluated statistically using analysis of variance as reported by McClave and Benson [16].

RESULTS AND DISCUSSION

Chemical Composition of Raw Materials and Pie: Data presented in Table 3 show gross chemical compositions of wheat flour 72% and the prepared pies. It is clear that adding lactic acid bacteria with whey protein to pie dough led to high level in protein, crude fiber, ash and decrease in fat and total carbohydrates content. The results also showed that energy values k cal/100g decreased in all pie samples in comparison to control due to the substitution of shortening by yoghurt.

Baking Quality and Freshness of Pie: The physical characteristics of the produced pie are presented in Table 4. There were no apparent differences in Pie volume and specific volume between all pie dough formulas and control sample. The changes in alkaline water retention capacity (AWRC) for different pie samples stored at room temperature for 0, 3 and 7days are shown in Table 4. It could be observed that the control sample had the highest value of AWRC, being 305, 297.6 and 290.4% at 0, 3 and 7days of storage, respectively. However, all pie formulas caused a noticeable increase in AWRC value at 0, 3 and 7days of storage compared with the control.

The data presented in Table 5 show the colour attributes of the cakes. With regard to the surface color, it was clear that the addition of lactic acid bacteria (*Lc. diacetylactis* and *Lb. plantarum)* did not appreciably affect the color quality. The coloring characteristics were more or less not markedly changed except for (a) character (redness to green) in most formulas and (L) lightness in all formulas. Even though differences among Hunter L, a and b values for cake batters were statistically insignificant, we observed that cake batters prepared with *Lc. diacytylactis* and *Lb. plantarum* were darker in color than the control.

Aroma Volatile Constituents of Fermented Milk with *Lactococcus Diacetylactis* and *Lactobacillus Plantarum* with 0.5 % Phenylalanine: Thirteen volatile compounds were isolated in headspace of fermented milk with (*Lactococcus diacetylactis* and *lactobacillus plantarum*) with 0.5% phenylalanine for each are cited with their area percentages as shown in Table 6 and Fig. 1 and 2. The compounds identified including 7 aldehydes, 1 alcohol, 2 ketones and, 2 esters and 1 acid. As shown in Table 6 the Strecker aldehydes 2-methyl butanal; 3-methyl butanal; phenylacetaldehyde and the two dicarbonyls 2, 3-butanedione and 2, 3-pentanedione were represented with remarkable concentrations. The formation of these compounds is mainly depending on the precursor amino acids [17] who reported that branched-chain amino acids (Leucine) aromatic amino-acid Phenylalanine and methionine being the main precursors of key aroma compound. Also data in Table 6 show 1-propanol represent with high concentration (9.53 and 10.91 %) in the samples and considered the second major compound. This alcohol derived from amino acids metabolism [18]. Acetaldehyde also found with remarkable concentration (5.41 and 8.26 %) in all samples. Acetaldehyde is one of the most common aldehydes found in dairy products.

Table 2: Flavor production.

Samples	Buttery	Ripe cheese	Cheesy	Yoghurt	Milk
Milk+ *Lc.diacetylactis*+0.5% phenylalanine	9±0.2	3±1.02	3±0.78	4±0.23	3±0.14
Milk+ *Lb.plantarum*+0.5% phenylalanine	3±0.03	8±1.02	9±1.45	4±0.45	4±0.35

Table 3: Proximate composition and energy value of raw and pie (On dry weight basis).

Samples	Moisture	Protein	Fat	Crude fiber	Ash	Total carbohydrate
Wheat flour	12.56±0.09	11.65±0.06	1.22±0.01	0.46±0.01	0.51±0.02	86.16±0.82
Formula 1	34.15±0.13	10.56±0.09	25.60±0.12	0.65±0.003	1.12±0.01	62.07±0.13
Formula 2	35.56±0.17	13.40±0.15	10.80±0.09	0.75±0.03	1.35±0.02	73.7±0.16
Formula 3	34.96±0.11	14.65±0.11	10.65±0.16	0.72±0.01	1.32±0.02	72.66±0.18

Table 4: Baking quality and freshness properties of produced pie.

	Baking quality			Water retention capacity(Freshness)		
Samples	Weight (g)	Volume (cc)	Specific volume	Zero time	3 days	7 days
Formula 1	120±0.65	370±0.68	3.08±0.03	305±0.36	297.6±0.22	290.4±1.16
Formula 2	122.2±0.62	380±0.45	3.11±0.01	315.0±0.35	305.8±0.63	295.3±1.25
Formula 3	121.8±0.13	375±0.38	3.08±0.05	320.0±0.56	308.6±0.85	295.5±0.96

Table 5: Hunte color parameter of pie.

	Pie					
	Crust			Crumb		
Samples	L	a	b	L	a	b
Formula 1	59.12±0.44	14.27±0.15	16.67±0.19	68.26±0.34	6.34±0.03	18.36±0.13
Formula 2	56.13±0.38	14.98±0.12	20.76±0.22	64.07±0.62	3.27±0.05	23.58±0.11
Formula 3	53.74±0.29	14.06±0.09	23.05±0.17	63.03±0.55	6.69±0.09	20.71±0.15

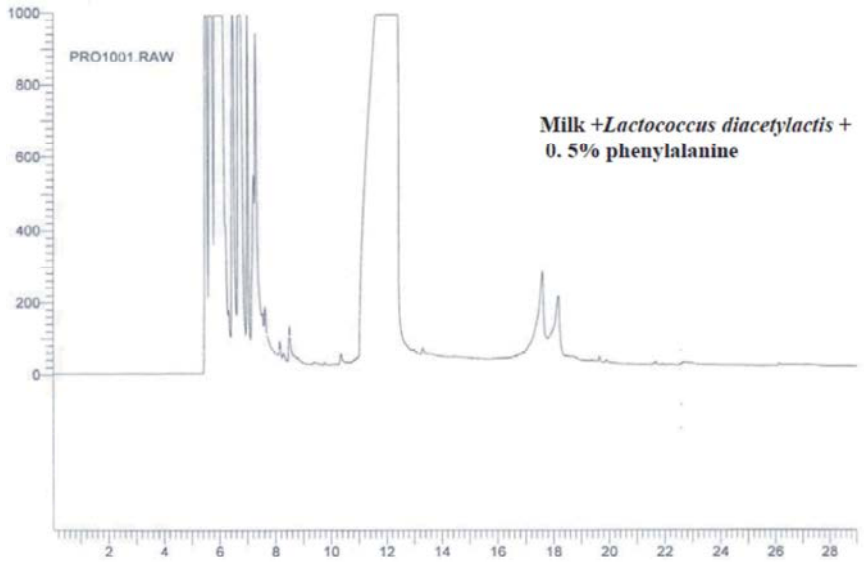


Fig. 1: Gas chromatograms of the volatiles isolated in headspace of fermented milk with *Lactococcus diacetylactis* and 0.5% phenylalanine.

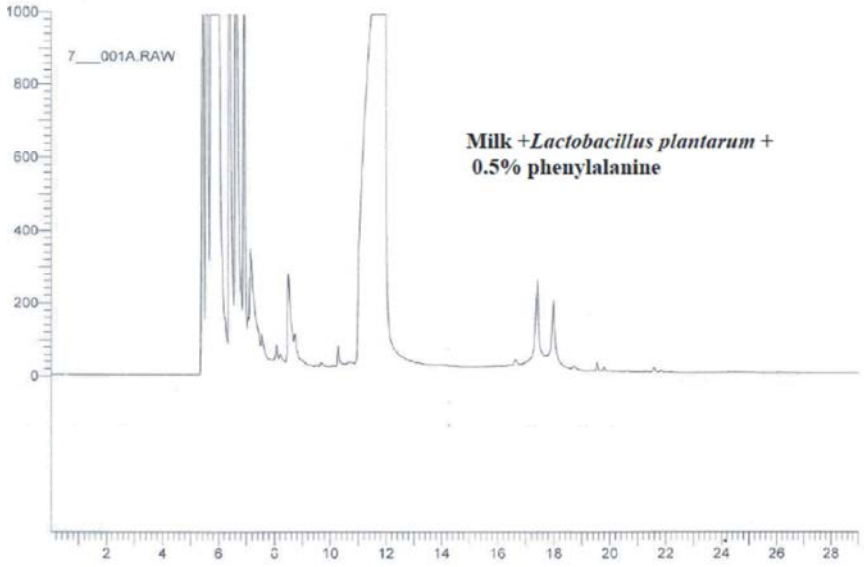


Fig. 2: Gas chromatograms of the volatiles isolated in headspace of fermented milk with *Lactobacillus plantarum* with 0.5% phenylalanine.

Data in Table 6 show two esters, ethyl butanoate and ethylhexanoate were identified in the samples where ethyl butanoate found with high concentration 65.28% and 60.6% in fermented milk with *Lactococcus diacetylactis* and *Lactobacillus plantarum*, respectively (Table 6). The importance contribution of esters to cheese aroma is not in doubt, since short-chain esters have a perception threshold to times lower than the alcohols from which they are derived [19].

Sensory Characteristics of Produced Pie: Results of the sensory evaluation of pie are shown in Table 7. The obtained results showed that, there were no significant differences in mouth feel, texture, break &shred, crust colour and symmetry shape scores between control pie and all different pie formulas. Taste and palatability of produced pie were improvement for formulas (with *Lc. diacytylactis* and *Lb. plantarum*) compared with control. This might be attributed to the

Table 6: Aroma volatile constituents of fermented milk with *Lactococcus diacetylactis* and *Lactobacillus plantarum* with 0.5 % phenylalanine.

			Area %		
Peak No.	K I[a]	Identified Compounds	Fermented milk + *Lactococcus diacetylactis* 5% Ph. Al	Fermented milk + *Lactobacillus plantarum* 5% Ph. Al	Methods of Identification[b]
1	581	Acetaldehyde	5.41	8.26	KI, MS
2	600	1-Propanol	9.53	10.91	KI, MS
3	616	2,3-Butanedione	3.17	5.46	KI, MS
4	630	2-Methyl butanal	2.22	4.15	KI, MS
5	638	3-Methyl butanal	5.48	ND	KI, MS
6	697	1-Butanal	0.88	0.45	KI, MS
7	729	2,3-Pentanedione	0.79	3.14	KI, MS
8	802	2-Hexenal	0.16	0.31	KI, MS
9	851	Ethyl butanoate	65.28	60.6	KI, MS
10	1003	Octanal	4.14	3.29	KI, MS
11	1016	Ethyl hexanoate	2.5	2.43	KI, MS
12	1039	Phenyl acetic acid	ND	0.1	KI, MS
13	1046	Phenyl acetaldehyde	0.05	0.16	KI, MS

a: Kovats index; Ph. Al: Phenylalanine ; ND-: not detected; b: Compounds identified by GC-MS(MS) and/or by comparison of MS and KI of standard compound run under similar conditions and Compounds listed according to their elution on DB5 column.

Table 7: Sensory characteristics of produced pie.

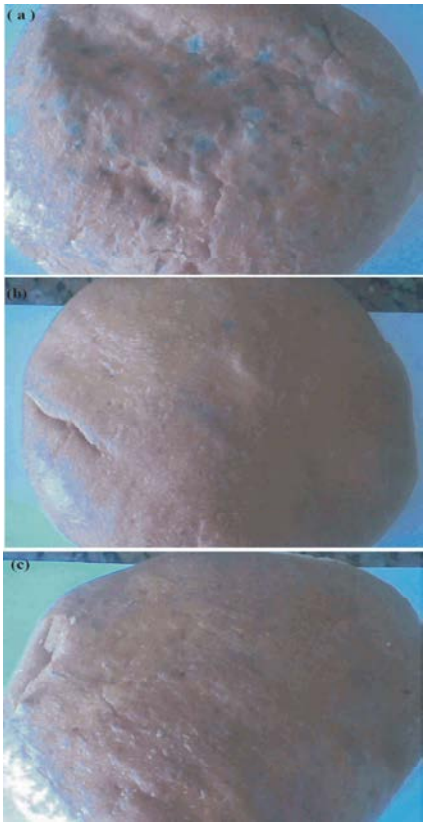
Samples	Taste (20)	Aroma (20)	Mouth feel (10)	Crumb texture (15)
Formula 1	16.2b±2.74	18.1a±1.66	8.4±1.17	13.3±0.82
Formula 2	15.8b±2.49	17.4a±1.03	7.9±0.99	13.1±0.74
Formula 3	16.0b±1.49	16.5b±0.88	7.6±0.52	11.9±1.79
LSD 0.05	1.753	1.47	NS	NS

Samples	Crumb color (10)	Break & Shred (10)	Crust colour (10)	Symmetry shape (5)
Formula 1	8.6a±0.84	8.4±1.34	8.7±0.82	4.4±0.52
Formula 2	8.2a±0.63	8.6±0.69	8.0±0.94	3.8±0.63
Formula 3	7.7a±1.06	8.7±1.34	8.0±1.05	4.2±0.79
LSD 0.05	0.873	NS	NS	NS

Table 8: Antifungal activity of lactic cultures.

	Diameter of zone of inhibition (mm)		
Lactic cultures	*Aspergillus flavus*	*Penicillium sp.*	*A. parasiticus*
Lactococcus lactis subsp diacetylactis	9	5	9
Lactobacillus plantarum	7	10	7

higher content in volatile aromatic or essential oils in these blends. Similar findings were also reported by Martinez-Castro *et al.* [19], Abd El-Mageed and Ragheb [20, 21], Whitfield *et al.* [22] and Liu *et al.* [23]. Flavour is one of the most valued sensory attributes in bread-volatile and non-volatile compounds produced during the fermentation of dough contribute to bread flavours. Reports show that the fermentation of dough with LAB can enhance the aroma and flavour [1, 2]. The growth of fungi is responsible for the formation of off-flavours and the production of mycotoxins; adding LAB to dough can prevent the growth of fungi and enhance the flavour of



(a) Control pie visible after 7 days.
(b) Pie with lactobacillus after7 days
(c) Pie with Lactococcus diacetylactis after7 days.

Fig. 3: Inhibition of molds by *Lactococcus diacetylactis* and *Lactobacillus plantarum* on pie samples after 7 days

bread. The produced compounds play an important role for any technological application to enhance the flavour, such as diacetyl which gives a buttery flavor. Results in Table 8 showed that *Lb. plantarum* and *Lc. lactis* subsp. *diacetylactis* inhibited *Aspergillus flavus, A. parasiticus* and *Penicillium sp.* Also, addition of LAB in fermented milk to pie dough confirmed previous results (Fig. 3).

In a previous study, screened 19 lactic acid bacteria strains for their antifungal activity against *A. parasiticus, A. fumigatus, Rhizopus stolonifer* and *Rhizopus* sp. They found *Lactococcus lactis subsp. diacetylactis* DRC1 and *Streptococcus thermophilus* 489 to be the most inhibitory strains to all fungal cultures [15]. Similar results were obtained for *A. flavus* by Reddy and Ranganathan [24], who tested cell free supernatant of *Lactococcus lactis subsp. diacetylactis* strain (S1- 67/C). The effect of LAB on mold growth and mycotoxin production, it appears that LAB have the potential to be used as biological control agents in foods to prevent mold growth in general and have specific antifungal activity against fungi isolated from bakery products. Moreover, the inhibition of spoilage and mycotoxigenic fungi by fermenting LAB could improve the shelf life of fermented products and reduce the risk of exposure to mycotoxins [25]. The shelf life of bread has been reported to be extended when certain LAB strains were added to bread formulations [1, 2]. The use of safe microbes in bread to extend the shelf life of the product is a great research area. Since LAB isolates are safe for use in foods, they are a significant alternative to chemical preservatives. Several researchers in the area of the bakery industry have successfully added LAB to dough and these strains grew well, producing the desired antifungal compounds in the dough. Various fungi isolated from bakeries were inhibited by *L. plantarum* (LB1) and *L. rossiae* (LB5) isolated from raw wheat germ. Organic acids and peptides synthesized during fermentation were responsible for the antifungal activity; formic acid had the highest inhibition activity [26].

CONCLUSION

LAB can be used as a starter culture or a co-culture in the bakery products industry to enhance the sensory properties of products and extend the shelf life. The antifungal compounds produced by LAB are important for the food industry for replacing or reducing the use of chemical preservatives. Future works should consider the use of the LAB supernatant as well as the cells because the active compounds can be present in the supernatant. A fermented dairy product is added to a bakery recipe to impart an enhanced flavour to the resulting bakery product, namely a slight acid and a cheese -like flavour.

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