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EFFECT OF *L. ACIDOPHILUS* 20552 AND *BIFIDOBACTERIUM LACTIS* BB12 ON ROPE-FORMING *BACILLUS SUBTILIS* ATCC 6633 SURVIVAL DURING THREE-STAGES OF WHEAT SOURDOUGH FERMENTATION

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ABSTRACT

This study aimed to investigate the survival of rope-forming *B. subtilis* ATCC6633 during three-stages of sourdough fermentation with or without *L. acidophilus* 20552 and/or *Bifidobacterium lactis* Bb12. Four strains of lactic acid bacteria are tested for their antimicrobial activities. All strains showed antimicrobial activity. *L. acidophilus* ATCC20552 recorded the highest inhibition zones, followed by *Bf. lactis* Bb12, while both of *L. acidophilus* ATCC4495 and *L. plantarum* ATCC14917 recorded moderate activity. Sourdoughs were made using *L. acidophilus* 20552 and/or *Bf. lactis* Bb12 with added *B. subtilis* ATCC6633 and fermented for 72h. Microbiological and physico-chemical analyses of doughs were periodically determined. Total bacterial, yeasts and molds counts (log_{10} cfu/g) gradually increased by developing doughs fermentation reached the maximum at the 3rd stage. Addition of LAB strains individually or in combination recorded lower total bacterial counts and higher yeasts and moulds counts than other treatments (negative and positive control). Same trend was observed with lactic acid bacterial counts. Starter treatments recorded the highest lactic acid bacteria than both of negative and positive controls initially and during all fermentation stages. Spore forming bacteria counts were sharply decreased by developing the fermentation stage till the end of fermentation in all starter treatments, while it gradually increased in positive control (with *B. subtilis* ATCC 6633 only) but not in negative control. At the end of fermentation stages spore forming bacteria were not detected in starter treatments. pH values of sourdoughs sharply decreased after the 1st fermentation stage (24hrs.), while at 2nd and 3rd stages it gradually decreased till the end of fermentation. Starter treatments recorded the lowest pH values during all fermentation stages. Total titratable acidity (TTA) sharply increased after the 1st fermentation stage, continued at the 2nd and 3rd stages, these increments were higher in starter treatments than both positive and negative controls reached the maximum at the end of fermentation. In conclusion, using three-stages fermentation method give *L. acidophilus* 20552 and *Bf. lactis* Bb12 the chance to completely inhibit rope-forming *B. subtilis* ATCC6633.

Key words: Sourdoughs, lactic acid bacteria (LAB), rope-forming B. subtilis, three-stages fermentation.

NTRODUCTION

All over the world, microbial attacks on bread cause very important losses in baking industry. Main reasons for these losses are the suitable conditions in bread and other flour based foods for microbial growth, such as a_w and pH. The frequent problem, occurring most frequently in baking industry is rope spoilage (Jenson, 1998). Bread can become contaminated with bacteria even under stringent conditions of production. *Bacillus subtilis*, *B.*

cereus, *B. megaterium* and *B. licheniformis* are the main organisms causing ropiness and arrive as contaminants from yeast, flour, etc. (Rosenquist and Hansen, 1995). When the cell counts of *B. subtilis* and *B. licheniformis* reach over 10^5 cfu/g, they present a potential risk of food borne illnesses (Kirschner and Von Holy, 1989). Rope can become noticeable within 12–24 h after loaf is taken from the oven. The spoilage is initially noticed as an unpleasant odour, followed by a discoloured, sticky soft bread crumb caused by the breakdown of starch and proteins by microbial amylases and proteases, and by the production of extracellular, slimy polysaccharides (Rosenquist and Hansen, 1995; Von Holy and Allan, 1990).

One potential way to prevent rope occurring in wheat bread is to control the growth and germination of *Bacillus* sp. already present; the occurrence of Bacillus spores in the raw materials used in baking is very common (Rosenquist and Hansen, 1995; Rocken, 1996). One of the additive-free methods to prevent rope formation is to use wheat sourdough. Sourdough bread is an ancient way of making high quality bread with a longer microbiological shelf-life and stronger flavour (Brummer and Lorenz, 1991; Rocken, 1996).

The antimicrobial activity of sourdough arises from lactic acid, acetic acid, carbon dioxide, diacetyl, ethanol, hydrogen peroxide and bacteriocins produced by lactic acid bacteria during fermentation. Bacteriocin-producing *Lactobacillus* strains are preferred as sourdough starter cultures because of their strong antimicrobial activity by bacteriocins (Rosenquist and Hansen, 1998; Vogel *et al.*, 1999). As well as the antimicrobial effects of sourdough, its addition improves dough properties, bread texture and flavour, retards the staling process and extends the shelf life. Sourdough addition is a promising procedure to protect bread from spoilage, since it is in agreement with the consumer demand for natural and additive-free products (Messens and De Vuyst, 2002). The antimicrobial substances produced by certain LAB can help to control the growth of spoilage and pathogenic organisms (Messens and De Vuyst, 2002).

Nowadays, sourdough process is used to produce traditional bread in some European countries and North America. It is also used in bread making to prevent spoilage and growth of pathogenic organisms and to improve bread aroma. Starter culture studies could be useful to minimize fermentation risks and to reach a standard production schedule. Starters composed of specific individual LAB, or mixed LAB and yeasts, became available a few years ago allowing the production of a full sourdough in a one-stage process. Such commercial starters improve the control of the sourdough production while ensuring reliable quality in bread production. The design of starters requires prior knowledge of the biochemical characteristics and baking potential of the microorganisms. Performance of LAB isolates has mainly been studied by characterization of the acidification properties such as pH, total titratable acidity (TTA) and lactic and acetic acids production during sourdough fermentation (Corsetti *et al.*, 1998; Hammes and Ganzle, 1998). In addition, traditional Greek wheat sourdoughs are made using a three-stage procedure and their microflora consist mainly of the yeast and the lactic acid bacteria (De Vuyst *et al.*, 2002]

In this study, the main purpose was studying the inhibition of growth rope-forming strain of *B. subtilis* ATCC6633 using LAB strains during three stages of wheat sourdough fermentation, and their ability to prevent the growth of *Bacillus subtilis* ATCC 6633 in wheat sourdough. For this reason, the antimicrobial activity of *L. acidophilus* ATCC20552, *L. acidophilus* ATCC 4495, *Bf. lactis* Bb12 and *L. plantarum* ATCC14917 against *B. subtilis* ATCC 6633 were tested using disc diffusion and agar spot methods. Microbiological and physicochemical analyses have been performed on sour-doughs during the fermentation period (72h).

MATERIALS AND METHODS

Bacterial strains and media

Lactobacillus acidophilus ATCC 20552, *L. acidophilus* ATCC4495, *L. plantarum* ATCC14917 and *B. subtilis* ATCC 6633 used in this study were obtained from American Type Culture Collection, while *Bifidobacterum lactis* Bb12 strain was obtained from Chr. Hansen laboratories, Copenhagen, Denmark. Lactic acid strains were grown on Man Rogosa Sharpe (MRS) medium at 37°C for 24 h. Bacillus strain were grown on Nutrient Broth (Oxoid) at 30°C for 24 h with shaking.

Determination of antimicrobial activity of LAB:

Agar spot and disc diffusion tests were applied to determine the antagonistic activity of LAB as described by Schillinger and Lucke (1989). Agar spot test was conducted by placing 0.5µl of an overnight LAB culture onto the MRS-0.2 agar and incubating for 24h at 25°C. For disc diffusion assay, the acidity of LAB cell-free supernatants that produced from the LAB cultures in MRS broth at 24 h at 25°C was adjusted to 6.5 with 10N NaOH and sterilized by filtering through a cellulose acetate filter with a pore size of 0.22µm.

Preparation of spore suspensions:

The *Bacillus subtilis* strain was inoculated into Nutrient Agar (Oxoid) supplemented with 10 mg/L of $MnSO4 \cdot \times H_2O$ and incubated at 30°C for 6days. Spores were collected with peptone buffered saline (PBS) and the cultures were heated at 85°C for 15 min in a water bath, then rapidly cooled with cold water to room temperature. The spore suspensions were centrifuged (7000g, 20 min, 20°C) and resuspended in PBS. The spore counts were determined by plate count agar (PCA) method at 30 °C for 72h (Katina *et al.*, 2002).

Preparation of LAB starter:

Actively growing single cultures of LAB were inoculated (inoculum level 1.0%, v/v) into Erlenmeyer flasks containing 100 ml of MRS broth, and incubated for 24 h at 25°C. Biomass was collected by centrifugation (5000 g, 15 min, 4°C) and resuspended into 50 ml of sterile saline. This cell suspension of LAB, that contained 10^9 to 10^{10} cfu/ml was used as a starter culture in sourdough preparation (Paramithiotis *et al.*, 2005)

Sourdough preparation:

For the preparation of the sourdough, a three-stage technique according to Paramithiotis *et al.* (2005) was applied. Dough 1 (D1) was prepared by mixing the 50ml of the starter culture with 400 g of wheat flour (82% extract) and 150 ml of tap water. Dough 1 was divided into two parts the first part was not inoculated neither with *B. subtilis* ATCC 6633 nor with lactic acid strains (fermented only with natural microflora) as negative control. The second part was inoculated with *B. subtilis* ATCC 6633 spores ($x10^4$ cfu/g), and extended divided to four parts; I: was not inoculated with lactic acid bacteria as positive control (fermented only with natural microflora); II: inoculated with *L. acidophilus* 20552; III: inoculated with *Bf. lactis* Bb12; IV: inoculated with mixed culture of two lactic acid strains.

After 24 h of incubation at 25°C, sourdough 1 (SD1) was formed. Then, 200 g of SD1 was mixed with 400 g of wheat flour and 200 ml of tap water to form dough 2 (D2). After 24 h incubation at 30°C, sourdough 2 (SD2) was formed. Then, 200 g of SD2 was mixed with 400 g of wheat flour and 200 ml of tap water to form dough 3 (D3). After 24 h incubation at 30°C, sourdough 3 (SD3) was formed. Sufficient dough and sourdough samples were collected at initial (after D1 formed) and at three-stages of fermentation. The microbiological samples were immediately refrigerated at 4°C till analysis (maximum 2 h), while physicochemical samples were kept frozen.

Microbiological analysis:

Ten grams of dough samples were homogenized with 90 ml of 0.85% (w/v) sterilized NaCl (Merck, Darmstadt, Germany) solution by using a blender. Further decimal

dilutions were prepared with the same diluents. Total aerobic mesophilic bacteria were enumerated on plate count agar (Merck, Darmstadt, Germany) following the pour-plate method and incubated at 30°C for 72 h (Lonner *et al.*, 1986). Yeast and moulds were determined on Potato Dextrose Agar (Oxoid, UK) acidified with 10% lactic acid (Merck, Darmstadt, Germany) to pH 3.5 following the surface plate method, with incubation at 25°C for 3 days. Lactic acid bacteria (LAB) were counted on MRS agar (Merck, Darmstadt, Germany) using the pour-plate method after incubating at 30°C for 3 days (De Man *et al.*, 1960). Spore-forming bacteria determined by heated the 10^{-1} sample dilution at 85°C for 15 min in a water bath then rapidly cooled with cold water at room temperature. The spore counts were determined by plating on plate count agar at 30°C for 72h (Katina *et al.*, 2002).

Physicochemical analyses:

Frozen sourdough samples were defrosted overnight in a refrigerator. The pH values of sourdoughs samples were measured according to AOAC, 1990. For the determination of total titratable acidity (TTA) the method described by Uluoz, (1965) was used where 10g sourdoughs sample were blended with 5ml acetone and 50ml distilled water were added to this mixture. This suspension was transferred into a beaker (200ml) by washing 45ml distilled water and titrated against 0.1 N NaOH to a final pH of 8.5. Total titratable acidity was expressed as the amount of NaOH used (ml).

RESULTS AND DISCUSSION

In this study, the inhibitory activities of four lactic acid bacteria strains (*Lactobacillus acidophislus* ATCC20552, *L. acidophislus* ATCC4495, *L. plantarum* ATCC 14917 and *Bifidabacterium lactis* Bb 12) against rope-forming *B. subtilis* ATCC 6633 were investigated on culture media to select the most potent antimicrobial strains, to be used individually or in combination during three stages of sourdough fermentation.

Utilization of untraditional strains of LAB in sourdough fermentation such as *L. acidophislus* ATCC 20552 and *Bf. lactis* Bb12 may be due to their antimicrobial activity. In addition, Simsek *et al.* (2006) isolated *L. acidophilus* strains from sourdough. Also, Palacios *et al.* (2008) applied human bifidobacteria strains in whole wheat dough fermentation. LAB strains may be preferred for sourdoughs starter cultures because they produced most of the total amount of acid and/or due to their high antimicrobial activities. High antagonistic strains can be effectively used in the sourdough system against the growth of *B. subtilis* (causing ropy fault) contaminated dough during fermentation as reported by Simsek *et al.* (2006).

Antimicrobial activity of lactic acid bacteria:

The antimicrobial activity of LAB strains was presented in table (1). The results indicated that all tested LAB strains showed antimicrobial activity against rope-forming *B. subtilis* ATCC6633. *L. acidophilus* ATCC 20552 recorded the highest antimicrobial activity using the two tested methods (20.67 and 6.00mm), followed by *Bifidobacterium lactis* Bb12 (21.33 and 5.83mm) for disc diffusion and agar spot methods, respectively, while both of *L. acidophilus* ATCC4495 and *L. plantarum* ATCC14917 recorded moderate antimicrobial activity against rope-forming *B. subtilis* ATCC6633.

These results are in compatible with those obtained by Simsek *et al.* (2006) who found that all lactic acid strains (included *L. acidophilus*, *L. sake*, *L. brevis* ssp. *lindneri* 2103 and *Pediococcus* sp.) isolated from sourdough exhibited inhibitory activity against *B. subtilis* ATCC6633 causing ropiness. In addition, all of the tested LAB strains were ineffective against *Saccharomyces cerevisiae* in both the disc diffusion and the agar spot tests (data not published).

Table (1): **Antimicrobial activity of lactic acid bacterial strains against rope-forming *B. subtilis* ATCC6633**

LAB strains	Inhibition zone diameter (mm)	
	disc diffusion	agar spot
L. acidophilus ATCC 20552	20.67	6.00
L. acidophilus ATCC 4495	18.33	5.17
Bifidobacterium lactis Bb 12	21.33	5.83
L. plantarum ATCC 14917	17.00	5.39

In similar baking investigations, various LAB strains isolated from sourdough exerted inhibitory activity against *B. subtilis* strains and prevented its growth (Rosenquist and Hansen, 1998).

Effect of lactic acid starter on microbial quality of sourdoughs:

Total microbial counts:

Data presented in table (2) showed the changes of total microbial counts of sourdoughs during three-stages of fermentation. The results indicated that total microbial counts (log_{10}) initially ranged between 6.10 to 6.54 log_{10} cfu/g and recorded higher numbers after the first 24h of fermentation (ranged between 8.00 to 8.78 log_{10}). Total microbial counts gradually increased by developing doughs fermentation reached the maximum at the 3rd stage (ranged from 8.49 to 8.81 log_{10}). These results are in agreement with those of Gül *et al.* (2005) who found that the log_{10} of total bacterial counts ranged from 5.97 to 9.57 log cfu/g. Also, these results were within the range obtained by Dıgrak and Ozcelik (1991), Gül (1999), and Simsek *et al.* (2006)

The high levels of total microbial counts may be due to increasing the yeast and lactic acid bacteria counts by developing the sourdough fermentation as shown in tables (3 and 4). These results confirmed by the results obtained with Vogel *et al.* (1999) who found that lactic acid bacteria are predominant microorganisms in sourdough and yeasts are present in significant numbers. Also, Mentes *et al.* (2007) found that yeasts and moulds and lactic acid bacteria increased by sourdoughs fermentation and recorded higher numbers.

Table (2): **Effect of lactic acid bacteria starter on total microbial counts (log_{10} cfu/g) during fermentation stages.**

Treatments	Fermentation stage			
	Initial	Sd1	Sd2	Sd3
Control negative	6.10	8.00	8.28	8.66
Control positive	6.54	8.78	8.71	8.81
*L. acidophilus*20552	6.32	8.47	8.48	8.49
Bf. Lactis Bb12	6.28	8.10	8.37	8.49
*L. acidophilus*20552+*Bf.lactis*Bb12	6.31	8.17	8.43	8.68

Starter treatments recorded lower total microbial counts than positive control treatment during 3 stages of fermentation, that may be due to inhibition of undesirable micro-organisms which may already present as microflora including *Bacillus* sp. as reported by Simsek *et al.* (2006) who reported that LAB inhibited inoculated rope-forming *B. subtilis* ATCC 6633. The highest total microbial counts recorded by positive control treatment may be due to its inoculation only with *B. subtilis* ATCC 6633 strain (without starter). Moderate counts were observed in negative control treatments.

Yeasts and moulds counts:

Yeasts and moulds counts (occurrence naturally in wheat flour) changes of sourdoughs during three-stages of fermentation presented in Table (3). The results indicated that few numbers of yeasts and moulds are present initially, that gradually

increased by developing the sourdoughs fermentation stage reached the maximum at the end of fermentation (3rd stage). This may be due to the produced acidity in the dough is more suitable for the growth of yeasts and moulds. The yeasts and moulds counts ranged from 5.59 (after 1st fermentation stage) to 6.68 (at the 3rd stage) $\log_{10}$ cfu/g. These results are within the range obtained by Gül *et al.* (2005) and Simsek *et al.*, 2006 who found that the $\log_{10}$ cfu/g of yeast and moulds counts of the sourdough samples ranged from 6.33 to 9.96 and 6.23 to 7.69 $\log_{10}$ cfu/g, respectively. Also, these results were within the range obtained by Dıgrak and Ozcelik (1991) and Gül (1999).

Table (3): Effect of lactic acid bacteria starter on yeasts and moulds counts ($\log_{10}$ cfu/g) during fermentation stages.

Treatments	Fermentation stage			
	Initial	Sd1	Sd2	Sd3
Control negative	2.25	5.59	5.97	6.22
Control positive	2.50	5.78	6.19	6.38
***L. acidophilus*20552**	2.33	6.08	6.37	6.68
***Bf. Lactis* Bb12**	2.21	6.01	6.35	6.60
***L. acidophilus*20552+*Bf.lactis* Bb12**	2.40	6.06	6.43	6.67

Addition of LAB strains individually or in combination recorded higher yeasts and moulds counts than other treatments (negative and positive control). *L. acidophilus* 20552 treatment recorded the highest yeasts and moulds count followed by the mixed culture of both *L. acidophilus* 20552 and *Bf. lactis* Bb12, then *Bf. lactis* Bb12 treatment. While, negative control followed by positive controls recorded the lowest yeasts and moulds counts, respectively. That may be due to the higher acidity and lower pH recorded in starter treatments and effect of LAB on yeasts and moulds encouragement. These results are in compatible with those obtained with Vogel *et al.* (1999) and Mentes *et al.* (2007) who found that when lactic acid bacteria are predominant microorganisms in sourdough, yeasts are present in significant numbers. Also, Simsek *et al.* (2006) found significant correlations between LAB and mould-yeast count. Moreover, when the LAB of sourdough samples were greater, also mould-yeast counts were greater.

Lactic acid bacteria count:

Data presented in table (4) revealed the changes in lactic acid bacteria counts of sourdoughs during three-stages of fermentation. The results showed that lactic acid bacteria were higher in all starter treatments at initial time than that of both of negative and positive controls (non inoculated), that may be due to starter addition. By developing the sourdough fermentation lactic acid bacteria gradually increased by increasing the fermentation stage of sourdough till the 3rd stage. The lactic acid bacteria counts were present in the same range obtained by Gül *et al.* (2005) who found that the LAB counts of sourdough samples were 5.28 to 9.66 $\log_{10}$ cfu/g. Also, these results were within the range obtained by Dıgrak and Ozcelik (1991), Gül (1999) and Simsek *et al.* (2006) (from 6.30 to 8.89 $\log_{10}$ cfu/g). In addition, lactic acid bacteria are predominant microorganisms in sourdough as reported by Vogel *et al.* (1999).

By comparing the treatments results revealed that, starter treatments (i.e. *L. acidophilus* 20552, *Bf. lactis* Bb12 and mixed culture of *L. acidophilus* 20552 and *Bf. lactis* Bb12) recorded the highest lactic acid bacteria counts than both of negative and positive controls during all fermentation stages, this may be due to higher initial counts in starter added treatments. Initially, LAB counts were very similar in each starter treatment with an average value of 6.32 $\log_{10}$ cfu/g (ranging from 6.17 to 6.43 log10 cfu/g). At the end of the sourdough fermentation 3rd stage, starter treatments recorded the highest LAB population ranging from 8.67 to 8.80 $\log_{10}$ cfu/g, while, positive control treatment recorded the lowest one (8.46 $\log_{10}$ cfu/g).

Table (4): Effect of lactic acid bacteria starter on lactic acid bacteria counts (log_{10}) during fermentation stages.

Treatments	Fermentation stage			
	initial	Sd1	Sd2	Sd3
Control negative	2.98	7.40	7.96	8.56
Control positive	3.41	7.65	7.90	8.46
*L. acidophilus*20552	6.37	8.28	8.29	8.80
Bf. Lactis Bb12	6.43	8.30	8.36	8.67
*L. acidophilus*20552+*Bf.lactis*Bb12	6.17	8.09	8.33	8.74

Spore-forming bacterial counts:

Spore-forming bacteria counts changed during three-stages of fermentation tabulated in table (5). The results indicate that spore-forming bacteria were present at low levels (1.08 log_{10} cfu/g) in negative controls (without added Bacillus), while all addition *B. subtilis* treatments recorded high numbers of spore-forming bacteria at initial time (ranged from 3.35 to 3.49 log_{10} cfu/g). By developing the fermentation stage spore forming bacteria counts were sharply decreased till the end of fermentation in all starter treatments, but it gradually increased in positive control, while slight increase was observed in negative control treatment. At the end of fermentation stages (3rd stage) spore forming bacteria were not detected in starter treatments but was present in negative and positive controls.

There was variation in the inhibition efficacy of the LAB strains even though the pH and TTA values of sourdoughs were comparable (Tables 6 and 7). The most effective growth inhibition of *B. subtilis* was seen with the sourdough made with mixed culture of both *L. acidophilus* and *Bf. lactis* Bb12, followed by *Bf. lactis* Bb12, then single culture of *L. acidophilus*, the amount of Bacillus was below the detection limit of 10 cfu/g. These results are confirmed with those obtained by Katina *et al.* (2002) who reported that in more acidic type of sourdoughs acidified with *P. pentosaceus, Lb. plantarum* or *Lb. brevis* the counts of natural and added Bacillus spores were reduced.

Table (5): Effect of lactic acid bacteria starter on spore-forming bacterial counts (log_{10}) during fermentation stages.

Treatments	Fermentation stage			
	Initial	Sd1	Sd2	Sd3
Control negative	1.08	1.75	2.14	3.10
Control positive	3.44	3.85	4.24	6.94
*L. acidophilus*20552	3.37	1.80	0.76	ND
Bf. Lactis Bb12	3.49	1.64	0.51	ND
*L. acidophilus*20552+*Bf.lactis*Bb12	3.35	1.38	0.23	ND

ND= not detect

The inhibitory effect of LAB against Bacillus strain was strongly dependent on the acidity level of sourdough. The low pH favours the undissociated form of weak acids which have been shown to be responsible for the antimicrobial effect (Alakomi *et al.*, 2000). On contrast, many investigators reported that the inhibition of rope forming *B. subtilis* was not due to the acidy or lactic acid, where Katina *et al.* (2002) found that when added lactic acid to final pH 4.9–5.0, did not prevent the growth of *B. subtilis*. This indicates that although the acidity level of sourdough is a major factor influencing the inhibitory effect of lactic acid bacteria (Voysey, 1990), there are also other factors are involved in the antimicrobial mechanism. Actually many earlier investigators reported that the inhibitory effects of organic acids (such as lactic) is mainly due to the undissociated portion of the acid and partially to H^+ concentration.

Also, the possible antimicrobial metabolites in sourdough might be bacteriocins or low molecular mass compounds with a wide spectrum of activity against Gram-positive and Gram-negative bacteria and fungi (Funel and Joyeux, 1993; Helander *et al.*, 1997). The inhibitory effect of LAB may result from the combination of lactic acid, low pH and other antimicrobial substances (low molecular mass compounds) present in the sourdough as reported by Katina *et al.* (2002). For these reasons sourdough is an essential ingredient for ensuring baking properties of doughs and preserving bread from spoilage (Messens and De Vuyst, 2002).

Effect of lactic acid bacteria starter on chemical properties of sourdoughs:

pH values:

Data presented in table (6) indicated that the pH values of sourdoughs gradually decreased till the 3rd stage of fermentation. After the 1st fermentation stage (24h.), the pH values of doughs samples sharply decreased from 6.0-6.23 at initial to values ranged from 4.32-5.76. By developing the fermentation stages (at 2nd and 3rd stages), the pH values gradually decreased till the end of fermentation and recorded values which ranged between 4.46 to 4.29 for 2nd stage and 4.38 to 4.20 for the 3rd one. These results are in the same average obtained by Dıgrak and Ozcelik (1991), Gul (1999), and Lonner *et al.* (1986) and were slightly higher than those reported by Simsek *et al.* (2006) and Paramithiotis *et al.*, (2005) who reported that the pH values of the successive sourdough preparations ranged from 3.50 to 3.94 and from 3.57 to 3.85, respectively. That may be due to different of starter cultures used by them.

The effect of the LAB starter on pH values was monitored over 72h sourdough fermentation and was compared with unionculated dough (negative and positive controls) prepared under the same conditions. At the beginning of the process (initial time), the pH values were quite similar for each treatments strain. During the first fermentation stage (first 24 h sourdough fermentation) the endogenous LAB microflora of the flour leading to a slight pH decrease at the end of first incubation period (sd1) for both negative (5.76) and positive (5.59) controls, respectively. The same trend was obtained by Robert *et al.* (2006). While, pH value of the starter treatments (inoculated with either individual or mixed LAB starter) sharply decreased at the same fermentation period (1st stage) and ranged between 4.35 to 4.32. The pH values decrement continued at the second and third fermentation periods (sd2 and sd3, respectively). Utilization of the mixed starter culture of *L. acidophilus* 20552 and *Bf. lactis* Bb12 has resulted clearly in a more rapid decrease of the pH value than when the inoculation was performed with either *L. acidophilus* 20552 or *Bf. lactis* Bb12 individually at sd2 and sd3. These results are confirmed with those obtained by Robert *et al.* (2006).

Table (6): Effect of lactic acid bacteria starter on pH values during sourdough fermentation stages.

Treatments	Fermentation stage			
	Initial	Sd1	Sd2	Sd3
Control negative	6.23	5.76	4.46	4.38
Control positive	6.19	5.59	4.44	4.45
*L. acidophilus*20552	6.10	4.35	4.32	4.30
*Bf. lactis*Bb12	6.07	4.32	4.31	4.28
*L. acidophilus*20552+*Bf.lactis*Bb12	6.00	4.33	4.29	4.20

The lowest pH values recorded in starter treatment may be due to highly LAB counts present in these treatments (table 4) as reported by Simsek *et al.* (2006) who found significant correlations between LAB count and pH values ($p < 0.05$). In addition, the variations between pH values of starter treatments in the range depending on the lactic acid bacteria strain as reported by Katina *et al.*, 2002.

Total titratable acidity:

Data presented in table (7) showed the total titratable acidity (TTA) of the sourdough during three-stages of fermentation. The TTA gradually increased by increasing the fermentation period till the 3rd stage. The TTA sharply increased after the 1st fermentation stage and recorded 3.0, 3.5, 10.5, 8.0 and 7.0 for negative control, positive control, *L. acidophilus* 20552, *Bf. lactis* Bb12 and mixed culture of *L. acidophilus* 20552 and *Bf. lactis* Bb12 treatments, respectively. By developing the fermentation (at 2nd and 3rd stages) the TTA continued increased, this increment were higher in starter treatments the both positive and negative controls reached the maximum at the end of fermentation (8.5, 10.5, 11, 11.0, 13.1 for negative control, positive control, *L. acidophilus* 20552, *Bf. lactis* Bb12 and mixed culture of *L. acidophilus* 20552 and *Bf. lactis* Bb12, respectively). These results are in agreement with those obtained by Dıgrak and Ozcelik (1991), Gul (1999) and Lonner *et al.* (1986). Also, the TTA values are in the same range obtained by Simsek *et al.* (2006) who found that acid values sourdough samples ranged from 7.6 to 19.3, and were higher than that reported by Katina *et al.* (2002) who found that, the TTA varied within the range of 3.9–7.8 depending on the Lactobacillus strain.

Table (7): Effect of lactic acid bacteria starter on titratable acidity during sourdough fermentation stages.

Treatments	Fermentation stage			
	Initial	Sd1	Sd2	Sd3
Control negative	1.0	3.0	8.0	8.5
Control positive	0.9	3.5	7.0	9.3
***L. acidophilus*20552**	1.3	10.5	10.5	11.0
***Bf. lactis*Bb12**	1.1	8.0	10.7	11.0
***L. acidophilus*20552+*Bf.lactis*Bb12**	1.2	7.0	12.0	13.1

The starter treatments recorded the higher TTA values, where it recorded 10.5, 8.0 and 7.0 after 24h (sd1), 10.5, 10.7 and 12.0 after 48h (sd2) and 11.0, 11.0 and 13.1 after 72h (sd3) for *L. acidophilus* 20552, *Bf. lactis* Bb12 and mixed culture of *L. acidophilus* 20552 and *Bf. lactis* Bb12, respectively; than both of negative and positive controls which recorded 3.0 and 3.5, 8.0 and 7.0, and 8.5 and 9.3 at same periods, respectively.

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تأثير بكتريا *L. acidophilus* 20552 و*Bifidobacterium lactis* Bb12 على بقاء *Bacillus subtilis* ATCC 6633 المكون للتحبل الخيطي أثناء ثلاث مراحل من تخمير عجينة القمح الحامضية

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تهدف هذه الدراسة لبحث بقاء ميكروب *Bacillus subtilis* ATCC 6633 المكون للتحبل الخيطي خلال ثلاث مراحل من تخمر عجينة القمح الحامضية بواسطة بكتريا *L. acidophilus* 20552 و*Bifidobacterium lactis* Bb12 كلا على حدة أوفي مخلوط. تم اختبار النشاط التضادي لأربعة سلالات من بكتريا حمض اللاكتيك وأظهرت كل السلالات نشاط تضادي، وسجلت السلالة *L. acidophilus* 20552 أعلى نشاط تثبيطي تلاها السلاله *Bf. lactis* Bb12 في حين أظهرت كلا من *L. plantarum* ATCC14917 و*acidophilus* ATCC4495 نشاط تضادي متوسط. تم عمل العجائن الحامضية باستخدام السلالتين ذات القدرة التثبيطية العالية (*L. acidophilus* 20552 و*Bf. lactis* Bb12) فرادي اوفي مخلوط مع إضافة *B. subtilis* ATCC 6633 وخمرت لمدة ٧٢ ساعة. تم تقدير الخصائص الميكروبيولوجية والطبيعية الكيماوية للعجائن دوريا. أظهرت النتائج ان العدد البكتيري الكلي وأعداد الخميرة والفطر ازدادت تدريجيا بتقدم تخمر العجينة ووصلت للحد الأقصى بعد المرحلة الثالثة للعجينة. وأدت إضافة بكتريا حامض اللاكتيك مفردة أومخلوطة لقلة العدد الكلي وزيادة أعداد الفطر والخميرة عن المعاملات الأخرى (الكونترول السالب والكونترول الموجب). وشوهد نفس الاتجاه بالنسة لأعداد بكتريا حامض اللاكتيك وسجلت معاملات البادئ أعلى أعداد منها عن الكونترول السالب والكونترول الموجب في البداية وخلال جميع مراحل التخمر. وفيما يتعلق بالبكتريا المكونة للجراثيم فقد أظهرت النتائج أن أعدادها انخفضت بحدة بتقدم مرحلة التخمر حتى النهاية في كل معاملات البادئ وعلى العكس فقد ازدادت أعدادها في معاملة الكونترول الموجب (تحتوى على *B. subtilis* ATCC 6633 فقط) ولكن ليس في الكونترول السالب. واختفت البكتريا المكونة للجراثيم في نهاية فترة التخمر بالنسبة لمعاملات البادئ فقط. وانخفضت قيم رقم الحموضة بشدة بعد المرحلة الأولى من التخمر (عد ٢٤ساعة) ولكن في المرحلة الثانية والثالثة قل معدل انخفاضها حتى نهاية التخمر. وسجلت معاملات البادئ أقل قيم لرقم الحموضة خلال كل مراحل التخمر. على العكس من ذلك ارتفعت قيم الحموضة الكلية المعايرة بشدة بعد مرحلة التخمر الأولى واستمر ارتفاعها خلال المرحلة الثانية والثالثة، هذه الزيادة كانت أعلى في معاملات البادئ عن الكونترول السالب والموجب ووصلت الحد الأقصى في نهاية التخمر. ويمكننا الاستنتاج ان استخدام طريقة مراحل التخمر الثلاث أعطت كل من *L. acidophilus* 20552 و*Bf. lactis Bb12* الفرصة لتثبط تماما ميكروب *B. subtilis* ATCC 6633 المكون للتحبل الخيطي.