Antimicrobial activity of lactic acid bacteria in rope producing strains of Bacillus from wheat bread

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Abstract: Contamination of bread by *Bacillus* species is a serious problem for the health of consumers, especially in the summer. A new way for preventing the growth of pathogenic bacteria is the use of bacterial interactions and its antimicrobial activities. The aim of this work is to detect Lactic acid bacteria produced antimicrobial substances that are effective against undesirable microbes. Flour samples of wheat varieties from Afghanistan (Lalmi-4 and Mazar-99) were collected in order to evaluate the antimicrobial activity of Femilex® (Lactic acid) and *Lactobacillus* species (isolated from kefir) against *Bacillus* strains. Counting of colony forming units (CFU) has been used as a traditional method to quantify *Bacillus* populations. The use of Lactic acid bacteria in biopreservation has great potential. The ability of Lactic acid bacteria to produce antimicrobial compounds such as nisin, which has the potential to inhibit the germination and growth of *Bacillus* species that cause food poisoning, is the reason for the preservation of these bacteria. The use of a medical product Femilex (Lactic acid) and *Lactobacillus* species originally isolated from kefir showed positive effect to prevent rope spoilage in bread.

Keywords: lactic acid bacteria, *Bacillus* counts, dough, bread and rope spoilage

INTRODUCTION

Ropiness is bacterial spoilage of bread that primary caused by *Bacillus subtilis* and occasionally *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus cereus* which as reported, originate from the raw materials, the atmosphere of the bakery and the surfaces of equipment [1]. Due to the food spoilage condition known as rope, bacteria belonging to the *Bacillus* genus have the potential to cause economic damage to the baking sector [2, 3].

Bacterial spores have highly differentiated, specialized types of cells that enable them to survive in unfavorable conditions (e.g. starvation, high temperatures, ionizing radiation, mechanical abrasion, chemical solvents, detergents, hydrolytic enzymes, desiccation, pH extremes and antibiotics). Due to their high resistance and ability to survive food processing and preservation procedures, spores may cause serious problems in the food industry [4,5]. It was reported that the use of chemical

preservatives (propionic and acetic acids) is one of the methods of inhibiting the germination and growth of *Bacillus* in bread, although currently there is a tendency to decrease the level of these substances [6, 7].

Lactic Acid Bacteria (LAB) were reported as being used as a starter culture or co-culture in the bread industry with success in terms of survivability in dough [8, 9]. LAB are utilized in food fermentations and have an antibacterial impact as a result of many metabolic activities (Lactose metabolism, proteolytic enzymes, citrate uptake, bacteriophage resistance, bacteriocin production, polysaccharide biosynthesis, and metal-ion resistance) [10, 11]. Several bacteriocins from Lactic Acid Bacteria have potential uses in food preservation, and their use in the food industry can help to reduce the amount of chemical preservatives added to foods as well as the intensity of heat treatments, resulting in foods that are more natural and richer according to organoleptic and nutritional properties [12].

MATERIALS AND METHODS

Samples

The flour samples of wheat varieties such as Lalmi-4 (L) and Mazar-99 (M) were collected from Baghlan Province, Afghanistan and transferred to the laboratory for determining the antagonistic activities of Lactic acid bacteria on rope spoilage of bread during dough processing.

Lactic Acid Bacteria as a starting culture

Two sources of LAB were used in this study. These starters were *Lactobacillus* species (isolated from kefir) and medical product Femilex® (Lactic acid). Each source was grown in MRS broth medium at 37°C for 24h under aerobic conditions.

The determination of rope spore counts in dough during processing

The amount of 13 g of each flour samples weighed out and under laboratory conditions in 10 ml of water that containing spore suspension of *Bacillus* species were added. During preparation of dough added separately 1,14 g Femilex® (L-1, M-1), 0,58 g Femilex® (L-2, M-2) and *Lactobacillus* of kefir (L-3, M-3) and control (L-4, M-4) to investigate the effect of starters on the experimental groups of dough.

Pro-baking 0, 1 g of each dough sample was weighed out and added in 10 ml sterile water then homogenized. The dough samples after 6 hours incubation, placed into oven. The baking time was 8-10 minutes at 240°C. After baking, the loaves were kept at room temperature for 30 min and 0, 1 g of each sample was weighed out and added in 10 ml sterile water then homogenized, after that the bread samples were placed in provocative terms: a loaf wrapped in a moist paper by putting in the package, and stored at 37°C. Post-baking in the samples during storage at 48 hours were determined rope spoilage onset organoleptically and microbiologically [13]. According to the protocol [14, 15], the samples aseptically inoculated on Tryptone glucose yeast extract agar (TGA). The samples were incubated at 37°C temperature for 1-2 days [16, 17].

The isolation and identification of *Bacillus* strains

For the identification of Bacillus strains contaminants bread have stored for two days, then seven colonies of *Bacillus* species like *Bacillus licheniformis* (two strains), *Bacillus subtilis* (two strains), *Bacillus megaterium* and *Bacillus cereus* (two strains) were isolated and investigated their thermal stability in the experiment *in vitro*: Colony suspension of contaminant in distilled water, heating in a water bath at a temperature of 97° C for 20 minutes and cultivated in Tryptone glucose yeast extract agar (TGA). Then that Colony suspension of contaminant in distilled water for 60 minutes at 97°C longer heated and then cultivated in TGA. The numbers of surviving spores were determined by the method of surface sowing in TGA on Petri dishes and concentration of spores in the various samples was calculated.

RESULTS

Effect of LAB on *Bacillus* species in dough processing

Flour samples were chosen to bake bread under laboratory conditions. The results showed that by adding Femilex® (Lactic acid) and *Lactobacillus* (isolated from kefir) in the dough during baking in the Mazar-99 sample caused death of the *Bacillus* population, while in the Lalmi-4 sample, their Population has been reduced to $0,1\times10^4$ CFU/mg and $0,2\times10^4$ CFU/mg respectively.

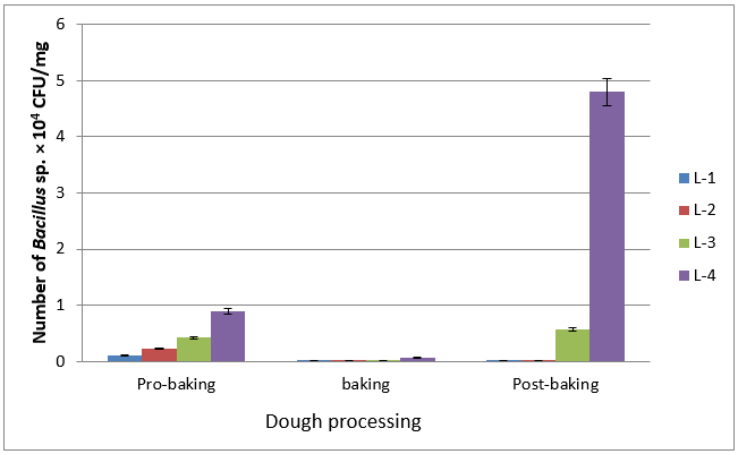


FIGURE 1. Effect of LAB on *Bacillus* species in dough (Lalmi-4) during processing

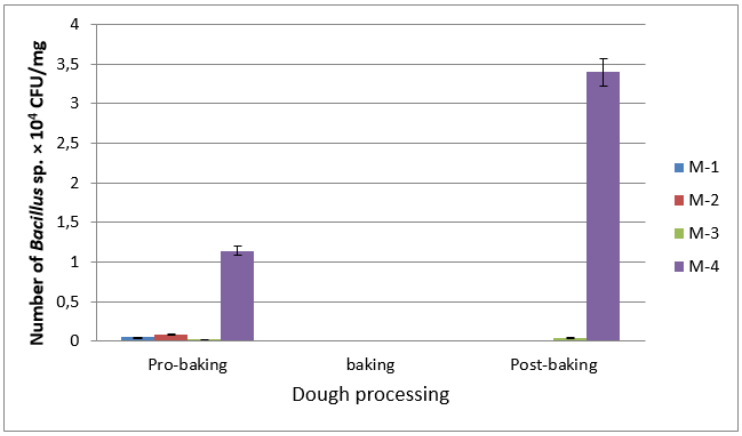


FIGURE 2. Effect of LAB on *Bacillus* species in dough (Mazar-99) during processing

After two days of storage, signs of ropiness, a specific ropy smell and soft, sticky to the touch crumbs were present in the breads made with the flour that harboured the highest *Bacillus* counts. The spore counts in this bread (L-3) was $5,6\times10^4$ CFU/mg. In the other samples of laboratory baked bread, there were no signs of ropiness but the *Bacillus* counts (Except M-1 and M-2) increased during the storage (Table 1).

Table 1.

Bacillus counts in laboratory-dough and baked bread

Flour samples used for baking	Number of *Bacillus*/mg		
	Pro-baking	Baking	Post-baking
L-1	1×10^4	$0,1\times10^4$	$0,1\times10^4$
L-2	$2,3\times10^4$	$0,1\times10^4$	$0,1\times10^4$
L-3	$4,2\times10^4$	$0,2\times10^4$	$5,6\times10^4$*
L-4	$8,9\times10^4$	$0,7\times10^4$	$4,8\times10^5$*
M-1	$0,5\times10^4$	0	0
M-2	$0,9\times10^4$	0	0
M-3	$0,2\times10^4$	0	$0,4\times10^4$
M-4	$1,14\times10^5$	$0,1\times10^4$	$3,4\times10^5$*

*Bread with rope symptoms.

Study of the thermal stability of bacteria-causative agents of rope spoilage of bread

From the analyzed samples of bread, seven rope producing Strains of *Bacillus* species were distinguished, which differed in the form of the colonies. In graphical form, death of the Bacterial pathogens of rope spoilage of bread at a temperature of 97 °C for 20 minutes and 60 minutes are shown in figure 3.

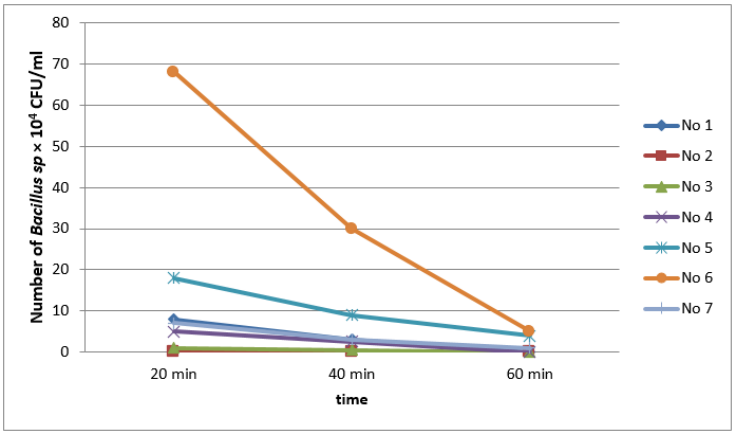
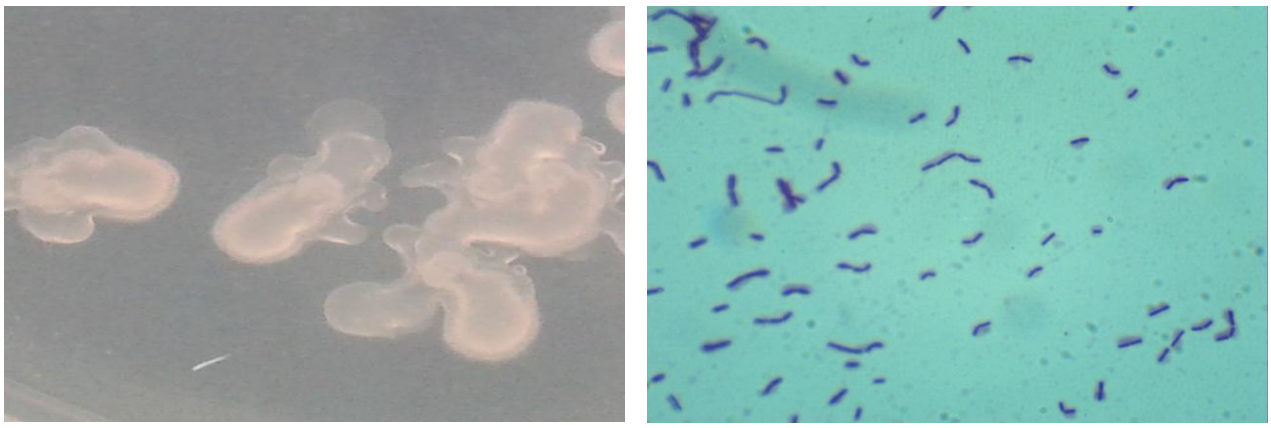


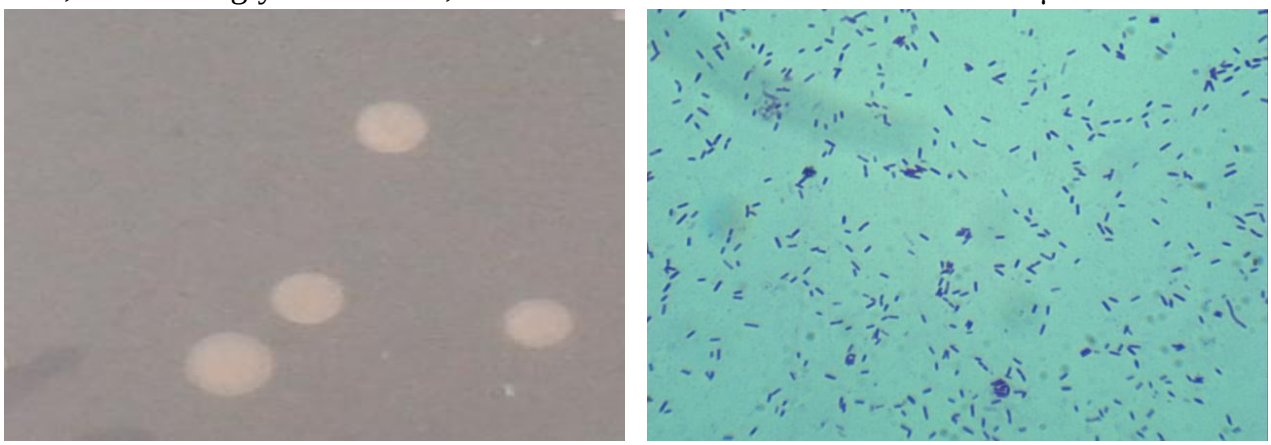
FIGURE 3. Curves of death of bacterial-causative agents of rope spoilage of bread at 97°C

The results obtained from the heat 97 °C at 20 minutes and 60 minutes, indicated that the heat for 20 minutes caused death of the bacteria colony No 2. while the rest of the colonies left alive. But the heat 60 minutes caused death of the bacteria colonies No 1, 3 and 4, and colonies No 5, 6 and 7 showed more heat resistance but there was rapid decrease in the number of bacterial colonies.

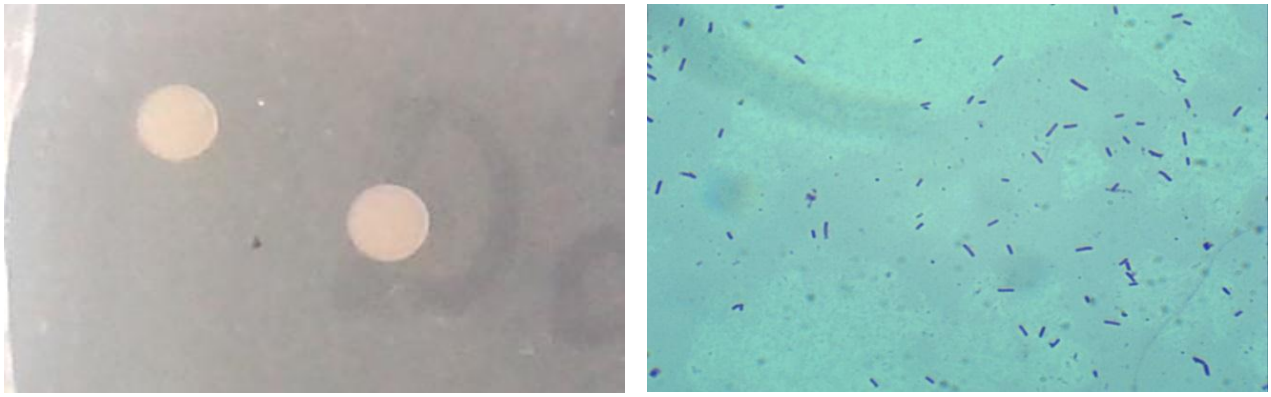
Pictures and microphotograph of rope producing bacteria that left alive after 60 minutes in the heat of 97 °C.



Colony No. 5: On the agar colonies showed as irregular form, had a cream color, adherent colonies and 3-7 mm in diameter. According to the morphological features - these were rods, located singly or in chains, the size of the cells was 0.6-0.8 x 1.5-3.0 μm.



Colony No. 6: Colonies were round with entire margin, showed a cream color, forming a drop of mucus on the surface and 1-3 mm in diameter. According to the morphological features – these also were rod shaped, located singly or in chains, the size of the cells was 2.0-5.0 x 1.2-1.5 μm.



Colony No. 7 Colonies generally appeared with dry, flat, round, whitish to cream with entire margin and 2-6 mm in diameter. According to the morphological features - these were rods, located singly or in short chains, the size of the cells was 1.0-1.2 x 3.0-5.0 μm.

FIGURE 4. Pictures and microphotograph of rope producing bacteria isolated from bread after 60 minutes of heating - colonies on an agar medium and cells under a microscope

Table 2.

Bacillus counts and heat resistance of spores of *Bacillus* spp. with the temperature adaptation of each species.

Colony No and name	CFU after 20 min / ml	CFU after 60 min / ml
1 (*Bacillus subtilis*)	8×10^5	0
2 (*Bacillus cereus*)	0	0
3 (*Bacillus licheniformis*)	1×10^5	0
4 (*Bacillus subtilis*)	5×10^5	0
5 (*Bacillus licheniformis*)	1.8×10^6	4×10^5
6 (*Bacillus megaterium*)	6.8×10^6	5×10^5
7 (*Bacillus cereus*)	7×10^5	1×10^5

DISCUSSION

Lactic acid bacteria play an important role in food industries and are used as starter cultures in the production of fermented products. The usage of sourdough containing Lactic acid bacteria (especially *lactobacillus*) has been common in the production of traditional breads.

Antimicrobial substances obtained from some Lactic acid bacteria are effective in controlling the growth of spoilage and pathogenic microorganisms and the use of such strains in the bakery industry is more importance. Therefore, this study have been conducted under the laboratory conditions, the result showed that the use of Femilex® (Lactic acid) and *Lactobacillus* species originally isolated from kefir have improved the microbiological safety of bread and other bakery products, because they can produce antimicrobial compounds such as nisin, which have the potential to inhibit germination and the growth of *Bacillus* species.

The oldest method of preparing sourdough, which is made by fermenting a mixture of flour and water, depends on spontaneous fermentation of flour natural flora. Alternately, the starter, a preenzyme containing one or more recognized species of lactic acid bacteria, may be where the lactic acid bacteria that develop in the dough originate [18]. Many researchers have investigated how the bacterial strain in sourdough affects the quality and longevity of wheat bread [19-21].

The present study showed that by using Lactic acid bacteria in bread production, the characteristics of dough, texture, aroma and taste of bread will be improved compared to bread made with baker's yeast. Also, by adding sourdough that contains Lactic acid bacteria, the shelf life of bread is increased and the appearance of mold and rope spoilage in bread is prevented.

Spoilage of bread by rope formation due to *Bacillus* species has been reported by several authors. Sorokulova [22] detected *Bacillus* in fresh baked bread from different samples of flour and ropy bread obtained from bakers, and identified as *B. subtilis* and *B. licheniformis* the isolates obtained from these samples. In a survey of [23] *Bacillus* isolated from white and whole meal wheat loaves were characterized as *B. subtilis*

(70%), *B. licheniformis* (24%), *B. pumilus* (2%) and *B. cereus* (2%). In our study the selected *Bacillus* isolates were micro-and macroscopically studied as well as using different fast morphological and biochemical tests. Analyses reveal that strains mainly belong to dominated species of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium* and *Bacillus cereus*.

CONCLUSION

The use of Lactic Acid Bacteria as a starting culture for bakery and food products can solve two problems; firstly, it can prolong the shelf life of the food products, which will reduce their cost and the need for low temperatures, secondly, it will satisfy the high requirement of modern consumers for high quality food that is free of chemicals. Above all, the product must be safe with an extended shelf life and good organoleptic properties. The proposed control measures, such as good raw material quality, clean bakery equipment, and strict temperature control during baking, may reduce the initial *Bacillus* spore counts.

Rope spoilage cannot be completely prevented by using only the recommended control procedures, such as analysis of the raw material, controlling the hygiene of the bakery equipment during baking, the use of an antirope starter consisting of selected LAB strains Femilex and *Lactobacillus* species originally isolated from kefir showed positive effect to prevent rope spoilage in bread.

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