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The effect of wheat bread contamination by the *Bacillus* genus bacteria on the quality and safety of bread

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Abstract

The aim of the study was to determine the level of contamination of white bread by aerobic spore-forming bacteria that may cause the ropiness and to evaluate the antimicrobial activity of lactic acid bacteria against aerobic spore-forming bacteria during the storage of wheat bread at different temperatures. The contamination of dough samples by aerobic spore-forming bacteria did not affect the quality of bread in the initial storage phase (6–16 h after baking). The critical *Bacillus subtilis* subsp. *spizizenii* ATCC 6633 levels that cause ropiness in baked bread were determined: 7.8×10^6 CFU (colony forming units) g^{-1} after storage of samples for 3 days at 2 ± 2°C, 1.3×10^6 CFU g^{-1} after storage of samples for 3 days at 18 ± 2°C and 6.0×10^6 CFU g^{-1} after storage of samples for 1 day at 30 ± 2°C temperature. It was determined that contamination of dough by aerobic spore-forming bacteria should not exceed 1.0×10^3 CFU g^{-1}. The evaluation of antimicrobial activities of lactic acid bacteria from *Lactococcus* and *Lactobacillus* genera against aerobic spore-forming bacteria was performed using an agar well diffusion method. In wheat bread, that had been fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus 148/3*, *L. acidophilus 336* and *L. casei* subsp. *casei*, the antimicrobial effects were observed for up to 3 days of storage at 18 and 30°C temperature. Weak signs of ropiness spoilage (sweet rope odour, discoloration of the crumb) were observed during the storage of the bread samples at 18 ± 2°C for 5 days (1.7×10^2 CFU g^{-1}) and at 30 ± 2°C for 3 and 5 days (1.5×10^2 CFU g^{-1}).

Key words: *Bacillus subtilis*, lactic acid bacteria, ropiness, wheat bread.

Introduction

Bacillus are abundant in various ecological niches: soil, water, insect and animal feces (Heyndrickx, 2011; Logan, Halket, 2011; Zhang et al., 2014). Harvested cereal grains also contain *Bacillus* spores (Needham et al., 2005; Fangio et al., 2010; Sakalauskas et al., 2014) as a result of processing or post processing contamination. These are mostly distributed on the surface of the grain. The dominant infective species usually are *Bacillus subtilis* (Voysey, 1989; Rosenkvist, Hansen, 1995), but the spores of *B. licheniformis*, *B. megaterium* and *B. cereus* may also be infective (Voysey, Hammond, 1993; Blackburn, 2006; Brul et al., 2011). Endospores of *Bacillus* are highly resistant to destruction during storage and thus they may survive in a dormant state and be transferred to processed products where they could become a problem. Previous studies (Sorokulova et al., 2003; Iurlina et al., 2006; Aydin et al., 2009) indicated that all types of wheat flour were contaminated with *Bacillus* spores. The incidence of wheat bread spoilage caused by *Bacillus* has increased presumably because more bread is produced without preservatives and with addition of raw materials such as bran and seeds. Ropiness, which is the most important spoilage of bread after mouldiness, occurs particularly in summer under warm and humid conditions and is mainly caused by *B. subtilis*. The dominance of *B. subtilis* in bread could be explained by the high heat resistance of this species – some spores may survive during the baking process, because the maximum temperature during baking in the center of loaf is 97°C to 100°C for a few minutes (Setlow, 2005). Surviving spores germinate if conditions are favourable; their vegetative cells multiply by decomposing proteins and carbohydrates of bread crumb, turning the bread pulp into a sticky, slimy, and foul smelling mass. This defect is characterized by an unpleasant sweet, musty smell of rotting pineapples (Mentes et al., 2007; Valerio et al., 2008; Kornacki, 2010). First signs of ropiness appear after 10–20 hours after baking. The colour of the bread loaf changes, it becomes soft and sticky, in later stages the bread crumb becomes more viscous. Bread texture deteriorates due to proteolytic and amylolytic enzymes secreted by *B. subtilis* (Sorokulova et al., 2003; Mentes et al., 2007; Valerio et al., 2008; Kornacki, 2010). The growth of *B. subtilis* is partially inhibited by a low water

activity and low pH in the bread pulp; however, under favourable development conditions; higher amounts of spores outgrow into vegetative cells and cause ropiness spoilage. Bread defects can cause huge economic losses for bakery industry (Thompson et al., 1998; Dewettinck et al., 2008). In order to prevent the development of ropiness defect in wheat bread, it is necessary to determine what level of bread contamination by aerobic spore-forming bacteria can cause the defect of ropiness, and what impact in this regard could have the duration of bread storage and temperature.

Lactic acid bacteria are widely used in the production of fermented foods as starter cultures. These bacteria are industrially important organisms and play an important role in food and feed fermentation and preservation either as the natural microflora or as starter cultures added under controlled conditions. Species used for food fermentation belong to the genera *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Lactobacillus*. Lactic acid bacteria have a long history of use in a variety of cereal fermentations, especially in the manufacture of baked goods (Hansen, 2002; Gobbetti et al., 2005).

The preservative action of starter cultures in food and beverage systems is attributed to the combined action of a range of antimicrobial metabolites produced during the fermentation process (Caplice, Fitzgerald, 1999; Denkova et al., 2014). Lactic acid is the dominant metabolite of lactic fermentation. The primary antimicrobial compounds produced by sourdough lactic acid bacteria are lactic and acetic acid, diacetyl, acetaldehyde, hydrogen peroxide, carbon dioxide and bacteriocins (Piard, Desmazeaud, 1992; Coloretti et al., 2008; Hladíková et al., 2012). Organic acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Ross et al., 2002). Bacteriocins produced by lactic acid bacteria are of great interest to the food fermentation industry as natural preservatives because of their ability to inhibit the growth of many food spoilage and pathogenic bacteria (Bredholt et al., 2001; McAuliffe et al., 2001).

The aim of the study was to determine the level of contamination of white bread by aerobic spore-forming bacteria that may cause the ropiness and to evaluate the antimicrobial activity of lactic acid bacteria against aerobic spore-forming bacteria during the storage of baked bread at different temperatures.

Materials and methods

Subjects of the research. The subject of the research was the wheat bread contaminated using the reference strain *Bacillus subtilis* subsp. *spizizenii* ATCC 6633. Lactic acid bacteria: *Lactobacillus delbrueckii* subsp. *bulgaricus 140/3*, *L. acidophilus 336*, *L. casei* subsp. *casei*, *Lactococcus lactis* subsp. *lactis 140/3*, *Lactobacillus bifidum*, *L. helveticus*, *L. plantarum* and *L. brevis* taken from the collection of microorganisms of Food Institute of Kaunas University of Technology were used for the examination of the antimicrobial activity. The research was conducted during 2012–2014 at the Food Institute of Kaunas University of Technology.

Preparation of spore suspension. Spore suspension was prepared from the reference strain *B. subtilis* subsp. *spizizenii* ATCC 6633. The culture was grown at 37°C on agar slants, using PCA (plate count agar) medium ("Liofilchem", Italy), then washed off from the agar with sterile saline (0.85% NaCl) solution. For contamination of bread dough, three different concentrations of spore suspensions were prepared: 1.4×10^4 (T-1), 2.8×10^6 (T-2) and 1.0×10^8 (T-3) CFU (colony forming units) ml^{-1}. For the determination of antimicrobial activity of lactic acid bacteria strains, the density of cell suspension was adjusted according to McFarland standard No. 0.5 (Cavalieri et al., 2005).

Contamination of bread by aerobic spore-forming bacteria. Samples of bread dough were contaminated by adding the spore suspension: 1) to the dough during mixing for 10 min, then the dough was kneaded for 5 min; 2) to the dry mixture of bread components, then the dough was mixed for 15 min; 3) to a liquid dough phase, then the dough was mixed for 15 min. In all the cases, the spore suspensions were dosed at 10 ml to each bread dough sample (370 g). While assessing the homogeneity of contamination of the samples by spores, the number of aerobic spore-forming bacteria was determined in four samples taken from the different points of the shaped bread dough. For contamination of bread dough by aerobic spore-forming bacteria (to get bread ropiness spoilage), the spore suspensions (three different concentrations) were dosed at 4, 8 and 12 ml for bread dough samples (370 g), and during dough preparation, to the liquid phase. For experiments, bread products were wrapped into parchment and nutritional (polypropylene) film, and stored for 5 days at 2 ± 2, 18 ± 2 and 30 ± 2 °C. While storing, the occurrence of ropiness spoilage was observed every day, and the aerobic spore-forming bacteria count was determined by plating into Petri dishes.

Wheat bread preparation techniques. The recipe of wheat bread is provided in Table 1.

Table 1. Wheat bread recipe

Ingredients	Amount g
Wheat flour, 812 D type	200.0
Vegetable oil	30.0
Sugar	40.0
Pressed yeasts	20.0
Salt	2.0
Water	206.0

Description of the technological process. The dough was prepared by a single-phase method, with 48.5% moisture content. All the ingredients of the recipe were mixed until homogeneous consistency for 15 min (5 min slowly and 10 min fast) in a sterilized spiral mixer Metos SP-100A/NH-B (Metos Manufacturing, Finland). The kneaded dough was matured at 33–35°C for 1.5 h. The acidity of the fermented and matured dough was 5.0–5.5°N. The initial rising of dough semi-products was performed at 22–24°C for 15–17 min. The dough samples were shaped manually. For experimental baking, hearth bread was prepared. The prepared dough was divided and risen in the shape forms. The duration of the last rising was 35–45 min, temperature – 33–35°C, relative humidity – 75–80%. The baking time was 20–25 min at 220°C. The baked bread was cooled to the room temperature. The quality characteristics for bread met the general requirements of the standard LST 1129:2003 Bread. General requirements.

Technological characteristics of bread quality. Bread crumb moisture content was determined by drying of a crushed sample at 130 ± 2°C (LST 1492:2013 Bakery goods – Methods for determination of moisture content). Bread acidity was measured by titration of the sample by a 0.1 mol l^{-1} NaOH solution (LST 1553:1998 Bakery goods and confectionery. Methods for determination of acidity and alkalinity). The bread porosity index was determined by using the Žuravliov equipment according to the standard LST 1442:1996 Bread, rolls and buns. Determination of porosity.

The antimicrobial activity of lactic acid bacteria strains. One millilitre of prepared suspension of *B. subtilis* subsp. *spizizenii* ATCC 6633 (1×10^5 CFU ml^{-1}) was added to 10 ml of the medium, melted before and cooled to 45°C and was mixed thoroughly. The prepared mixture of bacteria cell suspension and the medium were poured into 90 mm Petri dishes. After the medium had solidified and agar surface had dried, wells of 8 mm diameter were made in the plates and filled with 50 µl of lactic acid bacteria cultures solution. Antimicrobial effect against the bacteria cultures was evaluated after 24 h of growth (at the optimal temperature) according to the diameter of inhibition zones (in mm) around the wells. If inhibition zones failed to form around wells, the test solution had no antimicrobial effect against the tested bacteria culture.

The antimicrobial activity of supernatants from lactic acid bacteria cultures: *Lactococcus lactis* subsp. *lactis 140/3*, *Lactobacillus plantarum*, *L. casei* subsp. *casei*, *L. acidophilus 336*, *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus 148/3*, *L. brevis* and *L. bifidum* grown in MRS (De Man, Rogosa and Sharpe) ("Oxoid", England) broth at 32°C for 16 h was determined by an agar well diffusion assay. Supernatants were obtained by centrifugation of cultures at 10,000 g at 4°C for 10 min, adjusted to pH 6.2 with 1 moll^{-1} NaOH, sterilized through 0.22 µm pore-size (Millipore Corp., USA) and stored at 20°C until use. 50 µl aliquots of cell-free culture supernatants were poured into wells of 8-mm diameter cut in the solidified PCA agar plates previously inoculated (1×10^5 CFU ml^{-1}) with spores of *B. subtilis* subsp. *spizizenii* ATCC 6633. After 2 h at 4°C, the plates were incubated at 30°C temperature for 3 days. Then the antimicrobial activity of lactic acid bacteria was evaluated according to the clear zones formed around the well. The size of the clear zone indicated the sensitivity of the *B. subtilis* subsp. *spizizenii* ATCC 6633 to the tested lactic acid bacteria culture. The width of the growth-inhibition zone was measured using sliding calipers. After measuring the total diameter and excluding the diameter of the well, the width of the inhibition zone was calculated using the following formula:

$H = \frac{D-d}{2}$, where H is the width of the inhibition zone, mm, D – the total diameter of the sterile zone and the circle, mm, d – the diameter of the well, mm.

The lactic acid bacteria cultures with the highest antimicrobial activity were used for fermentation of wheat bread dough contaminated by spore-forming bacteria. Ropiness appearance was evaluated in the baked bread during 5 days storage at 18 ± 2 and 30 ± 2 °C.

Analysis of data. All tests were repeated three times. Data in all tables are presented as number of CFU g^{-1} except Table 2 (last column) and Table 3 where data are presented as mean and standard deviation. The distribution of the count of spore forming aerobic bacteria in different points of bread dough is compared by using Chi square test for quality of probabilities. The difference was considered as statistically significant if p value < 0.05.

Results

Bread dough samples were contaminated by aerobic spore-forming bacteria using three different methods: spore suspension was added to the dough, to the mixture of dry raw materials and to the liquid phase of the dough. The results of the study are presented in Table 2.

Table 2. Aerobic spore-forming bacteria count in bread dough for three different methods of sample contamination

Methods of sample contamination	Count of spore-forming aerobic bacteria in the different points of bread dough CFU g^{-1}				Assessment of the uniformity of distribution CFU g^{-1}	*p*-value
	A	B	C	D		
Spore suspension added to the dough	5.9×10^3	1.1×10^4	1.4×10^4	2.7×10^4	$(7.5 \pm 1.3) \times 10^4$	<0.001
Spore suspension added to the mixture of dry raw materials	3.9×10^2	4.9×10^2	1.5×10^2	7.9×10^1	$(2.8 \pm 1.9) \times 10^2$	<0.001
Spore suspension added to the liquid phase of the dough	1.4×10^4	1.1×10^4	1.9×10^4	1.4×10^4	$(1.5 \pm 0.3) \times 10^4$	>0.05

A – point in the cross-section of semi-product, at the point of height and width inter-section; B – point in the higher part (close to the surface) of the cross-section of semi-product; C and D – at random points of the longitudinal section line of the semi-product

The comparison of different methods of contamination by spore-forming bacteria showed that the homogeneity of distribution of these bacteria in bread dough differed. It was found that the spore suspension was distributed most evenly in the bread dough when added to a liquid dough phase: an average value of the spore-forming aerobic bacteria count was $(1.5 \pm 0.3) \times 10^4$ CFU g^{-1}. For further studies, this method of contaminating was chosen. Technological characteristics of bread (control and test samples, contaminated by different counts of aerobic spore-forming bacteria) were determined after wheat bread baking tests, i.e. 6 h after baking (Table 3).

It was found that the technological characteristics of the control and test samples differed insignificantly.

Table 3. Evaluation of the technological characteristics of bread quality

Samples	Technological characteristics		
	Moisture %	Acidity °N	Porosity %
Control	48.5 ± 1.7	5.4 ± 0.1	67.4 ± 1.4
T-1	48.8 ± 0.9	5.6 ± 0.1	65.4 ± 2.7
T-2	49.6 ± 0.4	5.5 ± 0.2	64.9 ± 2.1
T-3	49.8 ± 2.0	5.5 ± 0.1	65.7 ± 0.9

T-1 – the sample contaminated by 12 ml (1.4×10^4 CFU ml^{-1}) of spore suspension; T-2 – the sample contaminated by 12 ml (2.8×10^6 CFU ml^{-1}) of spore suspension; T-3 – the sample contaminated by 12 ml (1.0×10^8 CFU ml^{-1}) of spore suspension

This indicates that the contamination of the test samples by aerobic spore-forming bacteria in the initial storage phase (6 h after baking) did not affect the quality of wheat bread. During the storage of bread, the occurrence of ropiness defect was observed every day and the amount of aerobic spore-forming bacteria was determined. The results of the study on the ropiness defect in bread samples, contaminated by different amounts of spore suspensions (1.4×10^4, 2.8×10^6 and 1.0×10^8 CFU ml^{-1}) are presented in Tables 4–6.

No ropiness was observed in the samples of bread, whose dough was contaminated by different amounts of spore suspension (1.4×10^4 CFU ml^{-1}), stored from 1 to 5 days.

Table 4. Signs of ropiness defect in bread samples contaminated by different amounts of spore suspension (1.4×10^4 CFU ml^{-1})

Amount of spore suspension for contamination of sample ml	Temperature of bread storage °C	Appearance of signs of the ropiness defect			Aerobic spore-forming bacteria count in bread samples, CFU g^{-1}		
		storage of bread, days			storage of bread, days		
		1	3	5	1	3	5
0	2 ± 2	–	–	–	2.0×10^0	7.0×10^1	1.4×10^2
	18 ± 2	–	–	–	2.2×10^0	8.4×10^1	2.1×10^2
	30 ± 2	–	–	–	2.2×10^0	9.3×10^1	2.6×10^2
4	2 ± 2	–	–	–	3.0×10^0	2.2×10^2	9.7×10^2
	18 ± 2	–	–	–	4.0×10^0	2.5×10^2	9.7×10^2
	30 ± 2	–	–	–	4.0×10^0	2.5×10^2	9.7×10^2
8	2 ± 2	–	–	–	4.4×10^1	7.0×10^2	1.5×10^3
	18 ± 2	–	–	–	5.1×10^1	7.1×10^2	1.5×10^3
	30 ± 2	–	–	–	5.8×10^1	7.9×10^2	1.5×10^3
12	2 ± 2	–	–	–	5.7×10^1	2.0×10^2	4.8×10^3
	18 ± 2	–	–	–	6.1×10^1	2.4×10^2	2.6×10^4
	30 ± 2	–	–	–	5.9×10^1	2.8×10^2	3.3×10^4

Table 5. Signs of ropiness defect in bread samples contaminated by different amounts of spore suspension (2.8×10^6 CFU ml^{-1})

Amount of spore suspension for contamination of sample ml	Temperature of bread storage °C	Appearance of signs of ropiness defect			Aerobic spore-forming bacteria count in bread samples, CFU g^{-1}		
		storage of bread, days			storage of bread, days		
		1	3	5	1	3	5
0	2 ± 2	–	–	–	4.0×10^0	6.8×10^1	3.0×10^2
	18 ± 2	–	–	–	4.6×10^0	7.3×10^1	5.1×10^2
	30 ± 2	–	–	–	5.2×10^0	8.7×10^1	5.6×10^2
4	2 ± 2	–	–	–	7.1×10^1	1.2×10^3	1.6×10^3
	18 ± 2	–	–	–	7.4×10^1	3.2×10^3	3.0×10^3
	30 ± 2	–	–	–	8.0×10^1	4.0×10^3	3.0×10^3
8	2 ± 2	–	–	–	2.1×10^1	7.7×10^3	1.0×10^4
	18 ± 2	–	–	–	4.0×10^1	6.2×10^3	1.6×10^4
	30 ± 2	–	+*	++**	5.3×10^1	4.4×10^5	1.2×10^6
12	2 ± 2	–	–	–	1.9×10^1	7.1×10^2	4.7×10^4
	18 ± 2	–	++	+++***	2.1×10^2	2.0×10^6	1.2×10^7
	30 ± 2	–	++	+++	6.0×10^2	2.5×10^6	1.5×10^7

* – weak signs of ropiness spoilage (slight rope): faint odour, little mucus isolated, random black spots; ** – medium signs of ropiness spoilage (moderate rope): well perceptible odour, mucus on the surface of the pulp cover more than half cross-sectional area, brown and reddish spots; *** – distinct signs of ropiness spoilage (advanced rope): strong odor, much mucus of the pulp, reddish and purple spots

Table 6. Signs of ropiness defect in bread samples contaminated by different amounts of spore suspension (1.0×10^8 CFU ml^{-1})

Amount of spore suspension for contamination of sample ml	Temperature of bread storage °C	Appearance of signs of the ropiness defect			Aerobic spore-forming bacteria count in bread samples, CFU g^{-1}		
		storage of bread, days			storage of bread, days		
		1	3	5	1	3	5
0	2 ± 2	−	−	−	2.0×10^0	4.0×10^1	7.6×10^2
	18 ± 2	−	−	−	3.5×10^0	4.8×10^1	7.4×10^2
	30 ± 2	−	−	−	3.8×10^0	4.8×10^1	6.9×10^2
4	2 ± 2	−	−	−	1.7×10^2	6.4×10^2	1.9×10^3
	18 ± 2	+	++	+++	3.2×10^5	1.3×10^6	9.1×10^7
	30 ± 2	++	+++	+++++*	6.0×10^6	8.3×10^7	2.2×10^8
8	2 ± 2	−	+	++	2.1×10^5	4.5×10^6	3.8×10^7
	18 ± 2	+	++	+++	5.9×10^5	2.0×10^7	1.2×10^8
	30 ± 2	++	+++	++++	4.9×10^7	3.8×10^8	7.8×10^8
12	2 ± 2	−	++	+++	8.3×10^5	7.8×10^6	6.1×10^7
	18 ± 2	+	++	+++	1.0×10^6	1.1×10^8	4.3×10^8
	30 ± 2	++	+++	++++	1.4×10^8	6.3×10^8	1.8×10^9

* – very strong signs of ropiness defect (very advanced rope): distinct odour, the area of the cross-section is mucous, with bright purple spots

Samples contaminated by an average amount (8 ml) of spore suspension and stored at 30 ± 2°C showed weak signs of ropiness spoilage, which began to appear on the 3rd day ($N = 4.4 \times 10^5$ CFU g^{-1}), while on the 5th day, these signs were evaluated as moderate rope symptoms ($N = 1.2 \times 10^6$ CFU g^{-1}). The defects of ropiness increased after contamination of the samples with 12 ml of spore suspension: moderate rope symptoms appeared on the 3rd day ($N = 2.5 \times 10^6$ CFU g^{-1}), advanced rope symptoms, on the 5th day ($N = 1.5 \times 10^7$ CFU g^{-1}). The results showed that during the storage of bread samples for 3 days previously contaminated by 12 ml of spore suspension at 18 ± 2°C, the number of aerobic bacteria increased up to 2.0×10^6 CFU g^{-1} and moderate rope symptoms appeared, although the storage of samples was 5 days ($N = 1.2 \times 10^7$ CFU g^{-1}). In other cases of the study, the signs of ropiness defect did not appear in the bread samples contaminated by different amounts of spore suspension ($N = 2.8 \times 10^6$ CFU ml^{-1}) and kept from 1 to 5 days.

The data suggest that after contamination of bread samples with 1.0×10^8 CFU ml^{-1} spore suspension, the signs of ropiness appeared already from the first day of storage: weak signs at 18 ± 2°C, medium signs at 30 ± 2°C. During the storage of samples at 2 ± 2°C, weak and medium signs of ropiness appeared on the 3rd day of storage, depending on the amount of spore suspension. It was found that during the storage of samples for up to 5 days, the number of spore-forming aerobic bacteria increased evenly and the signs of ropiness were more intensive as the storage temperature increased. Prominent signs of ropiness defect appeared on the 5th day of storage of samples at 18 ± 2°C ($N = 9.1 \times 10^7 \div 4.3 \times 10^8$ CFU g^{-1}), or on the 3rd day at 30 ± 2°C ($N = 8.3 \times 10^7 \div 6.3 \times 10^8$ CFU g^{-1}); very distinctive signs appeared on the 5th day at 30 ± 2°C ($N = 2.2 \times 10^8 \div 1.8 \times 10^9$ CFU g^{-1}).

The antimicrobial activity of supernatants from lactic acid bacteria cultures: *Lactococcus lactis* subsp. *lactis 140/3*, *Lactobacillus plantarum*, *L. casei* subsp. *casei*, *L. acidophilus 336*, *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus 148/3*, *L. brevis* and *L. bifidum* was determined by an agar well diffusion assay. Antimicrobial activity of *Lactococcus* and *Lactobacillus* against aerobic spore-forming bacteria was different. *L. delbrueckii* subsp. *bulgaricus* (2.7×10^7 CFU ml^{-1}), *L. acidophilus* (1.0×10^5 CFU ml^{-1}), *L. casei* subsp. *casei* (4.1×10^7 CFU ml^{-1}) showed the highest antimicrobial activity – diameter of inhibition zone was from 5.8 ± 0.3 to 7.5 ± 1.3 mm (Fig.). *Lactococcus lactis* subsp. *lactis 140/2* (3.2×10^9 CFU ml^{-1}), *Lactobacillus bifidum* (1.1×10^9 CFU ml^{-1}) and *L. helveticus* (1.0×10^5 CFU ml^{-1}) was characterized by low (medium) antimicrobial activity against aerobic spore-forming bacteria (diameter of inhibition zone was <4.8 mm). *L. plantarum* (1.1×10^8 CFU ml^{-1}) and *L. brevis* (1.0×10^5 CFU ml^{-1}) had no effect against aerobic spore-forming bacteria (diameter of inhibition zone was from 1.3 ± 0.5 to 1.0 ± 0.0 mm).

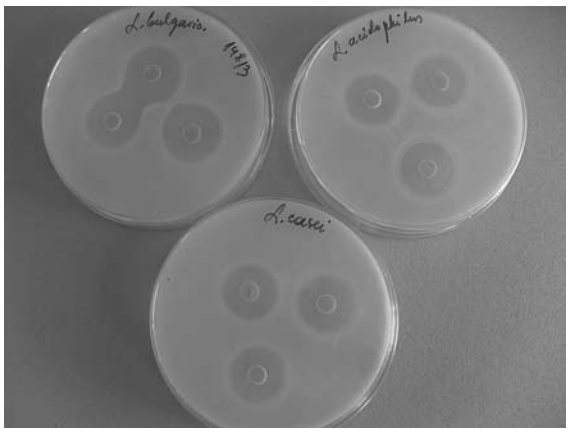

Figure. Antimicrobial effect of supernatants from *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. casei* subsp. *casei*

Wheat bread dough samples (contaminated with the critical number (1.0×10^9 CFU ml^{-1}) of aerobic spore-forming bacteria *Bacillus subtilis* subsp. *spizizenii* ATCC 6633) were fermented with the lactic acid bacteria characterized by the highest antimicrobial activity against aerobic spore-forming bacteria and the signs of ropiness in the baked bread were observed. It was found that the technological characteristics of bread dough samples in the initial stage of storage (6 h after baking) were complying with the regulatory requirements: pulp moisture – 49.7 ± 0.4%, pH – 5.1 ± 0.2 on, porosity – 71.8 ± 1.2%. In wheat bread samples, that had been fermented with lactic acid bacteria, the antimicrobial effects were observed at 18°C and 30°C temperature for up to 3 days of storage. Signs of ropiness were very weak during the storage of bread samples at 18 ± 2°C for 5 days (1.7×10^2 CFU g^{-1}) and during the storage at 30 ± 2°C temperature for 3 and 5 days (1.5×10^2 CFU g^{-1}).

Discussion

The data suggest that after contamination of dough samples by *B. subtilis* subsp. *spizizenii* ATCC 6633 4–12 ml 1.0×10^8 CFU g^{-1} (Table 6), the signs of ropiness (rotting smell, sliming of the pulp, coloured spots) appeared already from the first day of storage: slight rope at 18 ± 2°C, moderate rope at 30 ± 2°C. During the storage of samples at 2 ± 2°C, moderate rope appeared on the 3rd day of storage, depending on the level of contamination. The results of the research showed that during the storage of bread for up to 5 days, the number of spore-forming bacteria was still increasing evenly and the signs of ropiness became more intensive with increasing of storage temperature (Tables 6–5).

It was found that in the samples kept at a cooling temperature 2 ± 2°C (concentration of contamination 8.0×10^8 CFU g^{-1}), the initial signs of ropiness appeared on the 3rd day (in the bread samples N = $4.5 \times 10^6 \div 7.8 \times 10^6$ CFU g^{-1}), these signs were more prominent on the 5th day – N = $3.8 \times 10^7 \div 6.1 \times 10^7$ CFU g^{-1} (concentration of contamination 1.2×10^9 CFU g^{-1}) (Table 6).

Keeping the bread samples at bread consumption temperature 18 ± 2°C, the initial signs of ropiness were observed after one day and increased from 3.2×10^5 to 1.0×10^6 CFU g^{-1} (concentration of contamination 4.0×10^8 and 1.2×10^9 CFU g^{-1}). In other cases (at 18 ± 2°C) of the research, the slight rope appeared on the 3rd day – N = $1.3 \times 10^6 \div 2.0 \times 10^7$ CFU g^{-1}, and increased during storage: on the 5th day, the number of spore-forming bacteria in the samples increased N = $1.2 \times 10^7 \div 4.3 \times 10^8$ CFU g^{-1} (Tables 5–6). Experimental data suggest that the signs of ropiness in the bread samples appeared at 30 ± 2°C after one day of storage (N = 6.0×10^6 CFU g^{-1}) and increased with the increasing degree of contamination. Very advanced rope appeared at 30 ± 2°C in the bread samples on the 3rd–5th day of storage – N = $8.3 \times 10^7 \div 1.8 \times 10^9$ CFU g^{-1} (Table 6).

The critical levels of aerobic spore-forming bacteria, causing ropiness were determined: 1) 7.8×10^6 CFU g^{-1} after storage of samples for 3 days at 2 ± 2°C, 2) 1.3×10^6 CFU g^{-1} after storage of samples for 3 days at 18 ± 2°C and 3) 6.0×10^6 CFU g^{-1} after storage of samples for 1 day at 30 ± 2°C.

The results of this study showed that contamination of dough by aerobic spore-forming bacteria should be not higher than 1.0×10^3 CFU g^{-1}. The study also showed that the technological characteristics of bread dough samples (contaminated with the critical number of aerobic spore-forming bacteria *B. subtilis* subsp. *spizizenii* ATCC 6633) fermented with lactic acid bacteria characterized by the highest antimicrobial activity against aerobic spore-forming bacteria: *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *L. casei* subsp. *casei* in the initial stage of storage (6 h after baking) complied with the regulatory requirements: pulp moisture – 49.7 ± 0.4%, pH – 5.1 ± 0.2 on, porosity – 71.8 ± 1.2%. In wheat bread samples, that had been fermented with lactic acid bacteria, characterized by the highest antimicrobial activity, the antimicrobial effects were observed at 18°C and 30°C temperature for up to 3 days of storage. Signs of ropiness were very weak during the storage of bread samples at 18 ± 2°C temperature for 5 days (1.7×10^2 CFU g^{-1}) and at 30 ± 2°C temperature for 3 and 5 days (1.5×10^2 CFU g^{-1}).

Conclusions

1. Cereal grain and all types of wheat flour are usually contaminated with *Bacillus* spores from the environment. These spores may cause problems in bakeries, and one of the main defects of contaminated bread is ropiness.

2. The signs of ropiness appeared on the first day after contamination of dough samples by *Bacillus subtilis* subsp. *spizizenii* ATCC 6633 (4.0×10^8–1.2×10^9 CFU g^{-1}): slight rope appeared at 18 ± 2°C, moderate rope – at 30 ± 2°C temperature. During the storage of bread at 2 ± 2°C, moderate rope appeared on the 3rd day of storage. Consequently the number of spore-forming aerobic bacteria during the storage of bread for up to 5 days was still increasing evenly and the signs of ropiness increased with increasing of the storage temperature.

3. Lactic acid bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *L. casei* subsp. *casei* showed the highest antimicrobial activity. Very weak signs of ropiness were observed at 18°C and 30°C temperature for up to 3 days of bread storage: the amount of aerobic spore-forming bacteria was as low as 1.7×10^2 CFU g^{-1} during the storage of bread samples at 18 ± 2°C for 5 days, and 1.5×10^2 CFU g^{-1} during the storage at 30 ± 2°C for 3 and 5 days.

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Užterštumo *Bacillus* genties bakterijomis įtaka kvietinės duonos kokybei ir saugai

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Santrauka

Tyrimų tikslas – kvietinėje duonoje nustatyti sporinių aerobinių bakterijų skaičių, sukeliantį mikrobiologinės kilmės ydą – vadinamąją bulvinę ligą, ir įvertinti pieno rūgšties bakterijų antimikrobinį poveikį sporinėms aerobinėms bakterijoms esant skirtingoms temperatūroms kvietinės duonos laikymo metu.
Siekiant kontroliuoti gleivėjimo ydos plitimą kvietiniuose kepiniuose, tirtas ribinis sporinių aerobinių bakterijų *Bacillus subtilis* subsp. *spizizenii* ATCC 6633 skaičius, kvietinės duonos laikymo metu sukeliantis vadinamąją bulvinę ligą. Šiuo tikslu parinktas užkrėtimo aerobinėmis sporinėmis bakterijomis (*Bacillus subtilis*) būdas, atliktas iškeptų kvietinės duonos mėginių, kurių tešla prieš kepimą užkrėsta skirtingais kiekiais sporinių aerobinių bakterijų, mikrobiologinių ir technologinių kokybės rodiklių įvertinimas gamybos ir laikymo metu. Nustatyti ribiniai sporinių aerobinių bakterijų kiekiai, kvietinės duonos laikymo metu sukeliantys vadinamąją bulvinę ligą: $7{,}8 \times 10^6$ KSV (kolonijas sudarantys vienetai) g^{-1}, mėginius 3 paras laikant 2 ± 2 °C temperatūroje, $1{,}3 \times 10^6$ KSV g^{-1}, mėginius 3 paras laikant 18 ± 2 °C temperatūroje, ir $6{,}0 \times 10^6$ KSV g^{-1}, mėginius 1 parą laikant 30 ± 2 °C temperatūroje. Nustatyta, kad pradinis kvietinės duonos tešlos užteršimas sporinėmis aerobinėmis bakterijomis neturi viršyti $1{,}0 \times 10^3$ KSV g^{-1}. *Lactococcus* ir *Lactobacillus* pieno rūgšties bakterijų antimikrobinis poveikis sporinėms aerobinėms bakterijoms tirtas difuzijos į agarą būdu. Nustatyta, kad didžiausiu antimikrobiniu aktyvumu prieš sporines aerobines bakterijas pasižymėjo *Lactobacillus delbrueckii* subsp. *bulgaricus 148/3*, *L. acidophilus 336* ir *L. casei* subsp. *casei.* Sporinėmis aerobinėmis bakterijomis užkrėsti kvietinės duonos pusgaminiai buvo fermentuoti pieno rūgšties bakterijomis, pasižymėjusiomis didžiausiu antimikrobiniu aktyvumu. Iškeptoje duonoje įvertintas vadinamosios bulvinės ligos požymių pasireiškimas per 5 paras, duonos mėginius laikant 18 ± 2 ir 30 ± 2 °C temperatūroje. Bulvinės ligos požymiai (pakitęs kvapas ir minkštimo spalva) labai silpnai pasireiškė duonos mėginius 5 paras laikant 18 ± 2 °C temperatūroje (sporinių aerobinių bakterijų kiekis – $1{,}7 \times 10^2$ KSV g^{-1}) ir 3–5 paras laikant 30 ± 2 °C temperatūroje (sporinių aerobinių bakterijų kiekis – $1{,}5 \times 10^2$ KSV g^{-1}).

Reikšminiai žodžiai: *Bacillus subtilis*, kvietinė duona, mikrobiologinės kilmės duonos ydos, pieno rūgšties bakterijos.