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Solid-State Fermentation of *Silybum marianum* L. Seeds Used as Additive to Increase the Nutritional Value of Wheat Bread

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Summary

In the present study *Silybum marianum* L. seeds were fermented using solid-state fermentation (SSF) with several lactic acid bacteria (LAB) of *Lactobacillus* and *Pediococcus* genera, isolated from spontaneously fermented Lithuanian rye sourdough. A possibility to improve sensory properties (flavour) of *Silybum marianum* L. seeds using LAB fermentation was investigated. The composition of volatile compounds of the unfermented and LAB-fermented seeds of this plant was analyzed using gas chromatography-mass spectrometry (GC-MS). Fermented seeds have shown considerable differences mainly due to the accumulation of higher alcohols. Total amount of phenolic compounds, flavonoids and 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging activity of unfermented and fermented seeds were determined spectrophotometrically. The obtained results indicate that *Silybum marianum* L. seeds are a valuable source of bioactive compounds. The highest content of phenolic compounds and flavonoids (4596 and 1346 mg of rutin equivalents (RE) per 100 g, respectively) was determined in the seeds fermented with *Pediococcus acidilactici* KTU05-7 bacteria in solid-state fermentation. *Silybum marianum* L. seeds fermented with *P. acidilactici* KTU05-7 and *Pediococcus pentosaceus* KTU05-9 showed stronger antioxidant activity (1263 and 1041 mg of RE per 100 g, respectively), compared to the unfermented seeds (805 mg of RE per 100 g). The addition of *Silybum marianum* L. seeds fermented with *P. acidilactici* KTU05-7 bacteria had the highest effect on the decrease of the bacterial spoilage of bread. The aroma compounds from the fermented seeds extracted with supercritical carbon dioxide demonstrated the highest antimicrobial activity against the tested microorganisms. Ultrasonic pretreatment of the seeds reduced the total amount of microorganisms in the raw material. Microbiological tests revealed that the highest antimicrobial effect was achieved using the solid-state fermentation conditions. This study revealed that fermented *Silybum marianum* L. seeds are a suitable additive for natural flavouring of baked goods.

Key words: solid-state fermentation, lactic acid bacteria, *Silybum marianum* L., ultrasound, phenolic compounds, flavonoids, flavour compounds, antioxidant activity

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Introduction

Solid-state fermentation (SSF), which consists of the microbial growth and product formation on solid particles in the absence (or near absence) of water, has recently attracted special attention of researchers and manufacturers since it is more economical compared to the traditional microorganism cultivation in a liquid medium containing nutrients (*1–4*).

Several important factors have to be considered for the development of a successful bioprocess under SSF conditions. Some of the most important include the selection of a suitable microorganism strain and a solid substrate. A variety of microorganisms, including moulds, yeasts and bacteria may be used for SSF processes. Moulds and yeasts are the most common microorganisms used in the low water content fermentation systems, due to their ability to grow in such environments. Filamentous fungi have great potential to produce bioactive compounds by SSF, therefore, they are the most common microorganisms used for this purpose (*2,5,6*). They have also received great attention due to their ability to produce thermostable enzymes of high scientific and commercial value, such as amylases, pectinases, xylanases, cellulases, chitinases, proteases, lipases, β-galactosidases, *etc.* (*7,8*). However, the choice of the microorganism to be used in SSF depends on the desired end products. John *et al.* (*9*) demonstrated that lactic acid bacteria (LAB) like *Lactobacillus delbrueckii*, which are known for L-lactic acid production, can be used in SSF. In food fermentation processes, *e.g.* sourdough ecosystems, LAB (*10*) and yeast account for the dominant microflora and contribute not only to the development of the desired sensory properties of the final products but also to their microbiological safety, either as the natural microflora or as starter cultures added under controlled conditions. The structural genes of LAB are responsible for the antimicrobial activity of the strains. Until now, the main focus has been on LAB producing natural antimicrobials such as organic acids, hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids and reuterin. Over the last few years there has been an explosion of research on synthesized ribosomal and extracellular proteinaceous compounds, *i.e.* bacteriocins produced by LAB, which exhibit bactericidal and/or bacteriostatic modes of action against closely related species and even foodborne pathogens.

The main attention so far has been given to the application of residues, including coffee pulp and husk, sugarcane and agave bagasse, fruit pulp and peels, and corn cobs, among others, as supports and/or substrates for the production of enzymes by SSF (*11–14*), organic acids (*9,15,16*), antibiotics (*17,18*), flavour and aroma compounds (*19–21*), and bioactive compounds (*22,23*).

The SSF process variables including pretreatment, particle size of substrates, medium ingredients, supplementation of growth medium, sterilization of SSF medium, moisture content, water activity (a_w), inoculum density, temperature, pH, agitation and aeration have a significant effect on the efficiency of SSF processes (*24*). Among these, the moisture content and a_w have an important role in the SSF, and have been studied, described, and revised by several authors (*25–27*). Generally, the substrates have a water content ranging between 30 and 85 %. Lower values may induce the sporulation of the microorganism, while more elevated levels may reduce the porosity of the system, which can result in oxygen transfer limitations, which increases the risk of bacterial contamination (*28*).

Recently, considerable interest has arisen in the application of pulsed ultrasound technology as one of the few alternative technological means for the elimination of the bacterial contamination of fermentation media. High intensity pulses of sound waves have been shown to cause cavitations and collapse of microbubbles, which can be used to destroy several bacteria and degrade other pollutants (*29*). However, there is a lack of published data on the antimicrobial effect of pulsed ultrasound treatment on plant products and lactic acid bacteria cultivation processes in SSF. Furthermore, according to the literature, the use of SSF can increase the production of bioactive phenolic compounds (*30–33*).

The objective of this study is therefore to select optimal conditions of solid-state fermentation for the increase of the nutritional value of plant products using lactic acid bacteria (LAB) that can produce bacteriocin-like inhibitory substances (BLIS).

Material and Methods

Microorganisms and culture conditions

Lactobacillus sakei KTU05-6, *Pediococcus acidilactici* KTU-05-7 and *P. pentosaceus* KTU05-8, KTU05-9 and KTU05-10 previously isolated from spontaneously fermented Lithuanian rye sourdough and selected due to their preliminary inhibitory properties (*34*) were obtained from the culture collection of Kaunas University of Technology, Department of Food Science and Technology, Kaunas, Lithuania. Strains were stored at –70 °C in a Microbank™ system (Pro-Lab Diagnostics, Bromborough, Merseyside, UK) and were later propagated in the De Man-Rogosa-Sharpe (MRS) medium (Oxoid, Milan, Italy) at their optimal temperatures of 25 (KTU05-8 and KTU05-9), 30 (KTU05-6) or 35 °C (KTU05-10 and KTU05-7) for 24 h.

Plant material

As a model system the seeds of the milk thistle (*Silybum marianum* L. Gaertn.) were selected and analyzed. The plant was grown in an experimental field located in the Kaunas Botanical Garden of Vytautas Magnus University (KBG VMU, Kaunas, Lithuania) in 2011. The seeds were dried to a water content of approx. 7 % and stored in the dark at ambient temperature.

Preparation of fermented products

SSF was carried out at the Department of Food Technology of Kaunas University of Technology (KTU, Kaunas, Lithuania). In the first step, the seeds were ground in a laboratory scale impact mill MIAG (Bühler-Miag, Brunswick, Germany) to a particle size fraction of 0.5–2 mm. A mass ratio of 2 % (of total water and seeds) of freshly prepared LAB culture was mixed with sterile water and sprayed on freshly milled seeds, mixed and fermented at the optimal temperatures for LAB growth,

as previously mentioned, for 72 h. For the evaluation of sensory properties of wheat bread, *Saccharomyces cerevisiae* yeast was used as a starter material in the control sample instead of LAB during fermentation (at 30 °C for 72 h). Properties of the fermented product such as: total titratable acidity (TTA), pH, LAB growth and total number of microorganisms were determined during 24, 48 and 72 h of fermentation. The total phenolic and flavonoid content, the composition of phenolic compounds and quantitative and qualitative composition of volatile compounds, as well as total radical scavenging activity were determined for *Silybum marianum* seeds before and after 72 h of solid-state fermentation.

For the selection of optimal SSF conditions, the obtained seeds (before LAB fermentation) were pretreated by low frequency ultrasound (20 kHz). The treatment time for different samples varied from 0 to 480 s in steps of approx. 100 s. Fermented products were prepared by inoculating 2 % of the freshly prepared LAB starter to the seed. Fermentation was carried out for 72 h at an optimal temperature for each LAB. In this stage, the fermented products were used for the analysis of LAB content, and total content of microorganisms and fungi. Two control fermentations were carried out: traditional, with moisture content of 70 %, and the second control, with 60 % moisture content of the seeds.

Ultrasound treatment

The plant seeds were exposed to ultrasonic vibrations in the frequency range of 20–25 kHz to cause resonant vibrations in the seeds. The ultrasonic frequency around 20 kHz was chosen because in the range around this frequency about one quarter of the wavelength of flexural vibration is present along the seed's axis. In such a way, syneresis occurs and resonant vibrations are excited in the seeds. By sweeping the ultrasonic frequency in a small range, the vibrations of the whole seed mass are improved because in reality each grain is slightly different from another. The intensity of the ultrasound was around 120 dB, which corresponds to acoustic pressure of around 20 Pa. To achieve this kind of acoustic pressure, an ultrasonic cleaning vessel Pro'sKit® SS-802F (Prokits Industries Co., Ltd., New Taipei City, Taiwan, R.O.C.) was used.

Wheat bread making with fermented Silybum marianum seeds

The LAB strain *P. acidilactici* KTU05-7, which showed positive effect on sensory properties and increased the nutritional value of fermented products, was further tested to determine its antimicrobial activity during bread storage. Wheat bread samples were prepared using *P. acidilactici* KTU05-7 as a starter for fermented product preparation according to Juodeikiene *et al.* (*35*).

A simple formulation of wheat bread, consisting of wheat flour 100 g, compressed yeast 2 g, salt 1.5 g and water (required to reach 46 % dough moisture content), was used to reduce additional effects of other ingredients. Baking tests were carried out by adding 2 % of fermented and 1 % of dried *Silybum marianum* seeds to the bread formulations. The control baking test without additives was carried out at the same time. After baking, the bread samples were stored for 5 days (120 h) at room temperature. The quality evaluation of the loaves of bread was performed 24 h after baking. Antimicrobial analysis of bread was determined in bread crust and crumb 2 h after baking and after 5 days of storage.

Preparation of extracts

For spectrophotometric and high-performance liquid chromatography (HPLC) analyses, frozen or fermented samples of dry and fresh extracts of 0.1 and 0.5 g, respectively (the precise mass of dry material was determined for each sample) in 4 mL of aqueous methanol (1:3, by volume) were prepared using a Titertek microplate shaker (Flow Laboratories GmbH, Meckenheim, Germany) for 15 h. Extracts were filtered through a filter paper and 0.22-μm disposable Durapore PVDF membrane filter (Millipore, Merck KGaA, Billerica, MA, USA) before the analysis. The extracts were stored at 4 °C for further analyses.

Chemicals

HPLC grade methanol, acetic acid (99 %), sodium hydroxide and aluminium chloride were from Pliva-Lachema (Brno, Czech Republic). Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), HPLC grade acetonitrile and rutin were from Sigma-Aldrich (Seelze, Germany), while anhydrous sodium carbonate was from Eurochemicals (Bratislava, Slovakia). Hexamethylenetetramine (98 %) was from Fluka (Sigma-Aldrich, Buchs, Switzerland), and sodium acetate was from Thermo Fisher Scientific (Hampton, NH, USA).

Determination of total amount of phenolic compounds

Total amount of phenolic compounds was determined using a slightly modified Folin-Ciocalteu method (*36*). A volume of 100 μL of sample extract was mixed with 3000 μL of sodium carbonate solution (3.3 %) and 100 μL of Folin-Ciocalteu reagent. After 30 min of incubation at room temperature, the absorbance was measured at 760 nm using a spectrophotometer Spectronic 1201 (Milton Roy, Thermo Scientific, Waltham, MA, USA). Rutin (0.01–1.00 mg/mL) was used as standard for calibration. The TPC was expressed as rutin equivalents (RE) in mg per 100 g of dry matter.

Determination of total amount of flavonoids

The total amount of flavonoid compounds was evaluated according to a modified spectrophotometric assay using an aluminium chloride method (*37*). Stock solution consisted of 60 mL of pure methanol, 3 mL of acetic acid (33 %), 12 mL of hexamethylenetetramine (5 %), 9 mL of aluminium chloride (10 %) and 60 mL of previously prepared double distilled water. A volume of 80 μL of sample extract was added to 1920 μL of stock solution and mixed. After 30 min of incubation in the refrigerator (at 4 °C), the absorbance at 407 nm was measured. The calibration curve was prepared using rutin as a standard compound (0.01–1.00 mg/mL). Total flavonoid content was expressed as rutin equivalents (RE) in mg per 100 g of dry matter.

Determination of antioxidant activity

Antioxidant activity was determined using 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging capacity assay (*38*). The 100 mM sodium acetate buffer solution (pH=5.5) and DPPH radical solution (0.1 mM in acetonitrile and methanol, 1:1 by volume) were prepared before analysis. Equal volumes (250 mL) of buffer and radical solution were mixed and the absorbance was adjusted to 0.500 by adding acetate buffer. Sample extract (77 μL) was mixed with 3000 μL of DPPH solution. After 15 min of incubation at room temperature in the dark, the absorbance was measured at 515 nm. Control sample was prepared by mixing reagents and solvent without test compounds. To obtain the calibration curve, rutin solutions (0.05–0.25 mg/mL) were used. Antioxidant activity was expressed as rutin equivalents (RE) in mg per 100 g of dry matter.

Analysis of volatile compounds using GC-MS

Samples for gas chromatographic analysis were prepared using solid-phase microextraction (SPME). SPME device with Supelco 57750-U Stableflex^{TM} fibre coated with 65-μm PDMS-DVB layer (Sigma-Aldrich, St. Louis, MO, USA) was used for the preparation of samples. For extraction, 0.01 g of sample was placed in a 10-mL glass vial with the PTFE-lined silicone septa. The fibre was placed in the headspace of the sample at 25 °C for 1 h. The injection was performed by thermal desorption of the volatiles in the injection port of the gas chromatograph at 230 °C. For GC-MS measurements, a model GCMS-QP2010 gas chromatograph with a mass spectrometric detector (Shimadzu, Tokyo, Japan) was used. The ionization of the analytes was performed using an electron ionization mode at 70 eV. For the separation of volatiles, a low polarity Rtx®-5MS column (Restek Corporation, Bellefonte, PA, USA; length 30 m, coating thickness 0.25 μm, 0.25 mm i.d.) was used. Ion source temperature was set at 220 °C, and interface temperature was 260 °C. Sample injection was carried out for 1 min in order to ensure full desorption of volatiles from the SPME fibre. Split mode injection (1:10) was used. Temperature gradient program was set as follows: from 30 to 200 °C at 5 °C/min and up to 280 °C at 20 °C/min, then maintained for 2 min. The carrier gas was 99.999 % helium (AGA, Vilnius, Lithuania) with a pressure of 90 kPa at the column head, and the column flow of 1.61 mL/min. The compounds were identified according to the mass spectral library NIST v. 8.0 (The National Institute of Standards and Technology, Gaithersburg, MD, USA). Five identical samples were repeatedly measured for solid-phase microextraction coupled with GC-MC. The relative standard deviation (RSD) for peak area did not exceed 5.15 %.

Fermented product and bread quality evaluation

Total titratable acidity (TTA) and pH of the fermented products and bread crumbs were measured according to the method of AACC (*39*). The TTA value was expressed in mL of 0.1 M NaOH solution per 10 g of sample to obtain pH=8.5. The specific volume of the bread samples was evaluated as described by AACC (*39*). Sensory evaluations of the fermented products and loaves of bread were conducted by 15 panellists, consisting of KTU staff and students, using a 7-point rating scale for the odour of the fermented products and 8 bread attributes (bread odour, spice odour, bread flavour, spice flavour, acidity, bread firmness, springiness and moisture). Value of 1 corresponded to the lowest intensity and of 7 to the highest intensity of the attribute. The fermented products and bread were evaluated for overall acceptability using a 7-point hedonic scale where 7 is 'like extremely' and 1 'dislike extremely'. Coded samples were served and water was provided for rinsing between the sensory evaluations of the samples.

Seeds, fermented products and bread microbiological analysis

The effect of a novel fermentation medium on the growth of LAB was tested by determining their number in the newly prepared fermented products on the first, second and third day of fermentation. LAB counts were determined on MRS agar (Liofilchem, Roseto degli Abruzzi, Teramo, Italy) using plate count techniques. Plates were incubated for (72±4) h under anaerobic conditions (using atmosphere generation system AnaeroGen, Oxoid). The experiment was carried out in triplicate.

Microbiological contamination of primary seeds and fermented products was evaluated by calculating the total number of microorganisms and was expressed in colony-forming units per gram of product (CFU/g) using plate count agar (Liofilchem) according to LST EN ISO 4833:2003 (*40*).

The effect of fermented products on the microbiological characteristics of wheat bread was determined by counting the total number of microorganisms, fungal spores and the number of spore-forming aerobic mesophilic bacteria in bread crust and crumb 2 h after baking and after 5 days of storage at ambient temperatures (18–25 °C), and expressed in CFU/g.

The fungi were determined in yeast extract, glucose and chloramphenicol agar (ref. no. 610070, Liofilchem) after incubation at 25 °C for 5 days. The number of spore-forming aerobic mesophilic bacteria was determined on plate count agar (ref. no. 610040, Liofilchem) (before inoculation on plates, the diluted samples were heated for 10 min at 80 °C) after incubation at 30 °C for 3 days.

Supercritical carbon dioxide extraction and antimicrobial activity evaluation

Essential oils were extracted from *Silybum marianum* L. seeds using the supercritical carbon dioxide (99.9 % purity; AGA) extraction. For supercritical fluid extraction, an HP 7680T (Hewlett Packard, Palo Alto, CA, USA) apparatus was used. The following extraction conditions were used: CO_2 density of 0.3 g/mL, pressure of $88 \cdot 10^5$ Pa, temperature of the trap of 5 °C, extraction time of 15 min, volume of the extract fractions of 1.4 mL, CO_2 flow rate at the dynamic extraction of 1 mL/min, methanol as solvent, and 0.5 g of sample. The trap was filled with octadecylsilane-modified silica solid phase extraction particles. After trapping, the essential oils were desorbed by flushing solid phase adsorbent with methanol. The desorbed fractions were collected and kept in the refrigerator at 4 °C until GC–MS and antimicrobial analyses.

Antimicrobial activity of the extracts was tested against *Bacillus subtilis* ssp. *subtilis*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas gladioli* pv. *alliicola*, *P. cepacia*, *P. fluorescens*, *P. marginalis*, *P. facilis*, *P. aureofaciens* biovar. III, *P. cichorii*, *Aspergillus niger* and *Fusarium culmorum* indicator microorganisms, isolated from various food, according to the method described by Cizeikiene *et al.* (*34*).

Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as mean values±standard deviation. The significance of each instrumental measurement/descriptive attribute in discriminating between the samples was analyzed.

Results and Discussion

Quality and antimicrobial activity of fermented Silybum marianum products

The sensory properties of fermented products, characterized as intensity and acceptability of flavour, are important quality criteria. The seeds of *Silybum marianum* L. are a valuable source of bioactive substances. Lactic acid bacteria (LAB) are used for industrial food fermentation since they contribute to the preservation of the foods as well as to their texture and flavour (*41–43*). In this study the possibilities to improve the sensory properties (flavour) of *Silybum marianum* seeds using LAB fermentation was studied (Fig. 1).

The results of sensory evaluation of fermented products showed that the seeds of *Silybum marianum* L. provide a specific odour after fermentation, which is pleasing and acceptable to consumers. It has been noticed that after fermentation the quality and intensity of odour essentially change. In all cases the odour of fermented products was acceptable for consumers. However, some differences in the odour of fermented products using different LAB strains were noticed. The sweet and delicate odour was achieved when fermenting the seeds with one of the strains from the Department of Food Technology KTU collection – *L. sakei* KTU05-6; however, the intensity of the odour was low. Using other LAB strains for seed fermentation, malty odour characteristics were noticed. This study revealed that fermented *Silybum marianum* L. seeds could be a suitable additive for natural flavouring of baked goods. Interestingly, the yeast *Saccharomyces cerevisiae* used in traditional bread fermentation processes also gave an intensive and acceptable odour of the fermented plant products, typical of the cereal drink 'Kvas', which is traditional in Eastern Europe.

The results of total titratable acidity showed that TTA values of fermented products during 72 h of fermentation increased and obtained the highest values of 12.5, 13.3 and 14.8 when fermented with *P. acidilactici*, *L. sakei* and *P. pentosaceus* KTU05-10, respectively. The pH values during fermentation decreased and ranged from 4.7 to 5.0 after 72 h (Fig. 2a). The highest LAB number was observed at 48–72 h of fermentation (Fig. 2b). The acidification activity of LAB indicates most of the beneficial properties attributed to the fermented products. The main factor regulating acidification is the amount of fermentable carbohydrates (*44,45*), although the production of acids depends also on other parameters such as fermentation temperature and time (*46*).

Antimicrobial activity of the extracts of *Silybum marianum* L. seeds and fermented seed products is shown in Table 1. Essential oil extracts from *Silybum marianum* L. seeds show quite low antimicrobial activity only against *B. subtilis*, whereas the extracts prepared from fermented seeds with *Pediococcus acidilactici* KTU05-7 show inhibition radius up to 3 mm against *E. coli* and *P. gladioli* and lower inhibition against *B. subtilis*, *P. cepacia* and *P. aureofaciens*. Moreover, extracts from the seeds fermented with *P. pentosaceus* KTU05-10 show inhibition radius up to 5 mm against *P. gladioli* and *P. fluorescens* and somewhat lower inhibition against *B. subtilis*, *E. coli*, *S. typhimurium*, *P. cepacia* and *P. facilis*.

Optimisation of plant SSF process conditions

The microbiological analysis of fermented products under laboratory conditions revealed that the total microorganism counts (TMC) range from $74.5 \cdot 10^7$ to $89.5 \cdot 10^7$

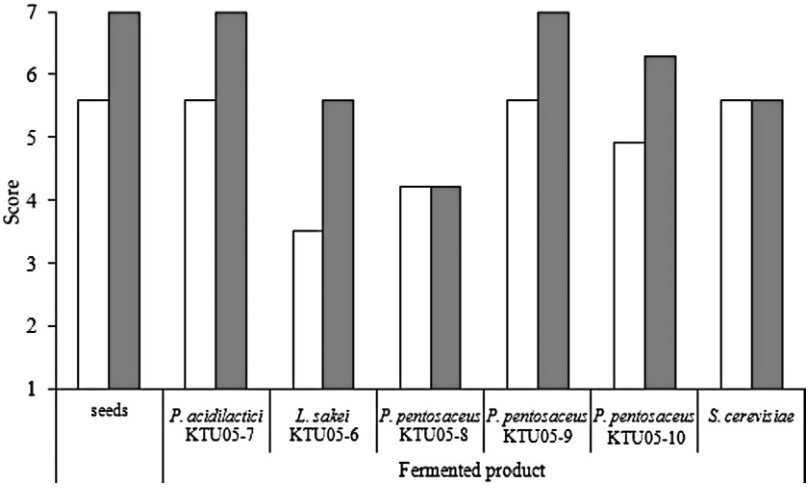

Fig. 1. The influence of SSF using different LAB strains (*Lactobacillus sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, KTU05-9 and KTU05-10) and *Saccharomyces cerevisiae* yeast on the intensity (□) and acceptability (■) of the odour of the bread samples

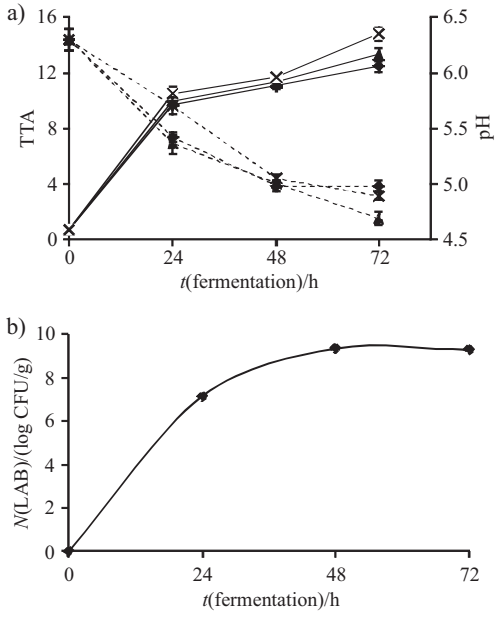

Fig. 2. The values of: a) total titratable acidity, TTA (line), and pH (dotted line) of fermented seeds during 72 h of fermentation: ▲ *Lactobacillus sakei* KTU05-6, ♦ *Pediococcus acidilactici* KTU 05-7, × *P. pentosaceus* KTU05-10; and b) *L. sakei* propagation in the newly prepared fermented products

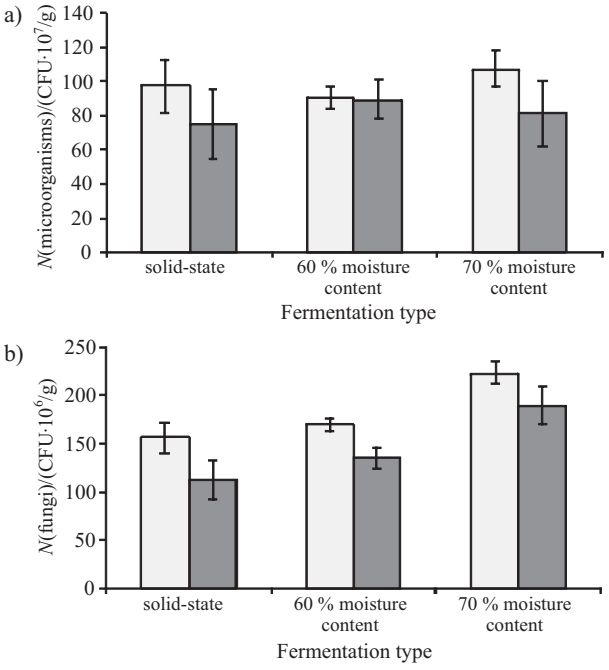

Fig. 3. The influence of fermentation on: a) total number of microorganisms and b) number of fungi. Microorganisms were counted before (□) and after (■) fermentation

Table 1. Antimicrobial activity of *Silybum marianum* L. unfermented and fermented seeds against indicator microorganisms

Sample	Indicator microorganism											
	B. subtilis ssp. *subtilis*	*E. coli*	*S. typhimurium*	*P. gladioli* pv. *alliicola*	*P. cepacia*	*P. fluorescens*	*P. marginalis*	*P. facilis*	*P. aureofaciens* biovar. III	*P. cichorii*	*A. niger*	*F. culmorum*
seeds	+	–	–	–	–	–	–	–	–	–	–	–
seeds fermented with *L. sakei* KTU05-6	+	–	–	–	+	–	–	–	–	–	–	–
seeds fermented with *P. acidilactici* KTU05-7	+	++	–	++	+	–	–	–	+	–	–	–
seeds fermented with *P. pentosaceus* KTU05-10	+	+	+	+++	+	+++	–	+	–	–	–	–

Inhibition radius without well diameter: +=inhibition zone 1–2 mm, ++=inhibition zone 2–3 mm, +++=inhibition zone 3–5 mm

CFU/g (Fig. 3). The TMC were approx. 8.6 and 16.8 % lower in SSF substrates than in the media in traditional (at 70 % moisture content) and the fermentation at 60 % moisture content, respectively. The number of fungal spore counts detected in SSF substrate were lower by approx. 40.6 and 16.7 % than in the traditional and fermentation medium at 60 % moisture content, respectively.

The tendency of the reduction of the TMC in fermented products was observed with the extension of the ultrasound treatment time of the raw seed material (Fig. 4a). The LAB numbers in different model systems of *Silybum marianum* L. seeds applying SSF, or fermentations at 60 and 70 % moisture content of the seeds are given in Fig. 4b.

The ultrasound treatment was effective in SSF, and in fermentations at 60 and 70 % moisture content. The reduction of CFU in SSF media within 90, 180, 280 and 380 s of ultrasound treatment was found by approx. 6.4, 40.5, 68.8 and 84.9 %, respectively, while applying traditional fermentation this parameter decreased by 24.0, 46.0, 68.8 and 80.0 %, respectively. Longer ultrasound treatment time (480 s) did not show significant influence on the reduction of microbiological contamination in the fermented products. This phenomenon can be explained by the impact of sound waves on extraneous microflora as well as the increased amount of antimicrobial compounds released from plant cells destroyed by ultrasound (*47*).

Furthermore, the effect of moisture content of fermentation media on the changes in LAB count was analysed (Fig. 4b). The microbiological analysis of the inoculated

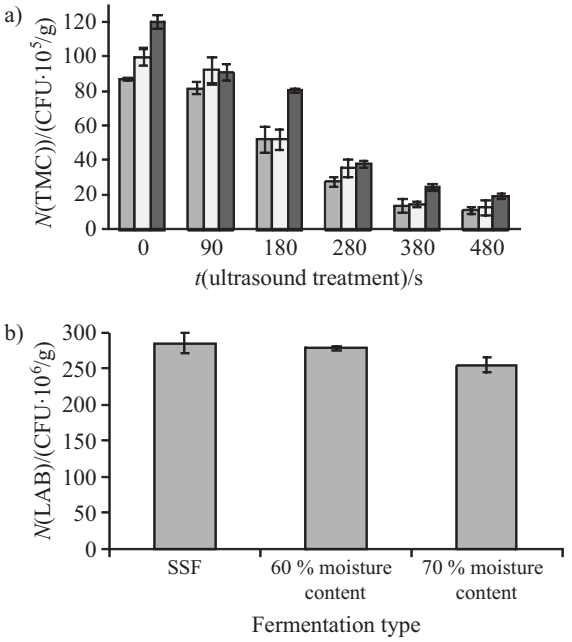

Fig. 4. The influence of ultrasound treatment time on: a) colony forming units of total microorganism content (TMC) per gram, and b) lactic acid bacteria (LAB) number in different model systems of *Silybum marianum* seeds applying SSF (■), fermentation at 60 (□) and 70 % (▩) moisture content. In the case of LAB counting, the seeds were treated with ultrasound for 480 s

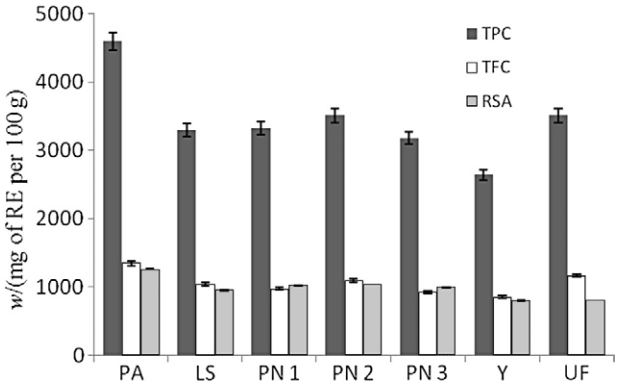

Fig. 5. Mass fraction of total phenolic compounds (TPC) and flavonoids (TFC), and radical scavenging activity (RSA) in *Silybum marianum* L. seeds unfermented (UF) and fermented with LAB: *Pediococcus acidilactici* KTU05-7 (PA), *Lactobacillus sakei* KTU 05-6 (LS), *P. pentosaceus* KTU05-8 (PN 1), KTU05-9 (PN 2), KTU05-10 (PN 3) and *Saccharomyces cerevisiae* yeast (Y) expressed as rutin equivalents (RE) in mg per 100 g of dry matter

LAB samples revealed that the count of *P. acidilactici* varied from $2.56 \cdot 10^8$ to $2.86 \cdot 10^8$ CFU/g. Most of the samples contained LAB cells within the range reported by other authors (*48–51*). The highest level of LAB ($2.86 \cdot 10^8$ CFU/g) was detected in the fermented products with a moisture content of 50 % (observed by SSF), while in the case of higher moisture levels (60 and 70 %) the detected LAB levels were slightly lower, $2.79 \cdot 10^8$ and $2.56 \cdot 10^8$ CFU/g, respectively. There is some controversy around the effect of the moisture content of fermentation media on LAB growth. Wang *et al*. (*52*) reported that lactic acid bacteria survive better at low water activity, while Hansen and Schieberle (*53*) stated that a medium with high water content was more appropriate for the LAB cultivation due to the solubility of nutrients in the medium. Nevertheless, microorganisms are found to be very sensitive to the changes in water content and nutrient concentration. The direct addition of selected starter cultures to raw materials is most important in the production of fermented foods, helping to control the overall standardization of the fermentation process and quality of the end product (*51*).

Influence of fermentation on nutritional value of Silybum marianum L. seeds

In our experiment different LAB strains producing bacteriocin-like inhibitory substances were used to study the effect of SSF on the total amount of phenolic compounds and flavonoids, and antioxidant activity of *Silybum marianum* L. seeds. As Fig. 5 demonstrates, the influence of SSF on the content of biologically active compounds is dependent on the type of microorganisms (LAB or yeast) and the used LAB strain. The highest level of phenolic compounds and flavonoids was obtained using the fermentation of seeds with *P. acidilactici* KTU05-7 (4596 and 1346 mg of RE per 100 g, respectively), while using yeast the total content of these components decreased during fermentation from 3512 (in the raw material) to 2638 and from 1167 to 851 mg of RE per 100 g. *Silybum marianum* L. seeds fermented using SSF with *P. acidilactici* KTU05-7 and *Pediococcus pentosaceus* KTU05-9 showed a stronger antioxidant activity (1263 and 1041 mg of RE per 100 g, respectively) than unfermented products (805 mg of RE per 100 g), which was probably related to the markedly higher contents of phenolic acids, flavonoids and aglycone isoflavone with more free hydroxyl groups formed during SSF (*54*). Another different LAB strain such as *L. sakei* KTU05-6 used in SSF was not effective for the improvement of bioactivity and antioxidant properties of *Silybum marianum* L. seeds.

The results of GC-MS/SPME analysis are presented in Table 2. The compositions of volatile compounds of the unfermented *Silybum marianum* L. seeds (reference) and LAB-fermented products of this plant have shown considerable differences. Both quantitative and qualitative differences have been observed. Fermented products mainly differed in the accumulation of higher alcohols. It is evident that different compounds predominate in the reference sample and in the fermented samples. The main compounds found in the headspace of fermented *Silybum marianum* L. seeds were as follows: isopentyl alcohol 55.6 %, *n*-hexanol up to 23 % and butyl carbinol (amyl alcohol) which varied from 5 to 13 %, unlike in seeds fermented with yeast, where the amount of the last compound increased to 21 %. Interestingly, there was no ethanol determined in the headspace of the *Silybum marianum* L. seeds fermented with yeast, which was the case in the fermentation of some other medicinal plants (results not shown). This could be explained by the low content of sugars in *Silybum marianum* L. seeds. Butyric (butanoic) acid is another product better known for anaerobic fermentation, which was determined in the samples fermented with some LAB, namely *L. sakei*, *P. pentosaceus* KTU05-8 and KTU05-10. In the unfermented sample, several essential oils were determined. The highest mass fraction, calculated as chromatographic peak area fraction

Table. 2. The composition of volatiles from *Silybum marianum* L. seeds (unfermented or fermented with various strains)

Compound name	RT/min	*w*(volatile)/%						
		P. acidilactici KTU05-7	*L. sakei* KTU05-6	*P. pentosaceus* KTU05-8	*P. pentosaceus* KTU05-9	*P. pentosaceus* KTU05-10	Yeast	Unfermented
isopentyl alcohol	3.446	43.15	47.11	22.54	18.43	18.23	55.65	3.20
butyl carbinol	3.486	4.40	13.77	5.22	9.86	5.10	21.01	n.d.
butyric acid	4.475	n.d.	3.56	9.24	n.d.	6.31	n.d.	n.d.
2-methyl pirazine	5.295	n.d.	n.d.	n.d.	3.43	n.d.	n.d.	n.d.
n-hexanol	6.475	23.67	11.66	15.29	12.95	15.80	n.d.	2.57
α-thujene	8.096	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.87
benzaldehyde	9.163	2.51	n.d.	11.89	10.77	1.72	n.d.	n.d.
n-hexanoic acid	9.888	n.d.	n.d.	n.d.	n.d.	2.94	n.d.	n.d.
2-pentyl furan	10.121	2.48	1.16	n.d.	3.69	1.85	n.d.	n.d.
nonane	10.348	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.52
p-cymene	11.136	3.31	1.87	5.35	6.30	3.89	1.45	27.68
limonene	11.264	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.29
benzyl alcohol	11.493	n.d.	1.38	n.d.	n.d.	2.80	n.d.	n.d.
γ-terpinene	12.232	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.20
phenethyl alcohol	13.976	n.d.	2.35	n.d.	n.d.	4.87	n.d.	n.d.
carvone	17.966	4.81	4.51	9.87	11.16	8.78	3.37	n.d.
thymoquinone	18.150	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.00
carvacrol	19.640	n.d.	1.28	n.d.	n.d.	2.24	n.d.	2.54
β-elemene	22.174	12.49	8.51	20.59	20.17	17.92	6.15	21.25
longifolene	22.602	3.19	1.67	n.d.	n.d.	3.92	n.d.	7.68
	total	100.00	98.82	100.00	96.76	96.38	88.62	84.81

n.d.=not determined, RT=retention time

of the sum of all integrated peak areas in the chromatogram, was of *p*-cymene and β-elemene (27.68 and 21.25 % respectively). Limonene, α-thujene, thymoquinone, γ-terpinene, longifolene and carvacrol were determined at lower mass fractions, which did not exceed 8 %. Some essential oils such as limonene, α-thujene and γ-terpinene were not present. Considerable amounts of carvone were formed during the fermentation with different LAB and yeast, particularly with all *P. pentosaceus* strains (over 8 %). Carvone belongs to the family of terpenoids. It is found naturally in many essential oils and dominates in the essential oils from seeds of caraway (*Carum carvi* L). The majority of *R*-(–)-carvone used in commercial applications is synthesized from limonene. During fermentation, it can be formed by oxidation of *p*-cymene, the amount of which was considerably reduced. In contrast, another major volatile β-elemene was almost fully retained during the fermentation with all *P. pentosaceus* strains. Conversion of phenylalanine to benzaldehyde initiated by an aminotransferase from *Lactobacillus* and chemical degradation of intermediates to a flavour compound benzaldehyde is well known (*55*). Benzaldehyde is a colourless liquid, which provides a characteristic pleasant almond-like odour. It is the primary component of bitter almond oil and can be extracted from a number of other natural sources. It is the simplest aromatic aldehyde and one of the most used in industry. High mass fractions of benzaldehyde were determined as a result of fermentation with *P. pentosaceus* KTU05-8 and KTU-05-9 strains (over 10 %) and a lower amount was present in the samples fermented with *P. pentosaceus* KTU05-10 and *P. acidilactici* (1.72 and 2.51 %, respectively). The chemical composition of volatiles indicates that some original flavour characteristics are retained after fermentation, although the mass fraction of essential oils is lower.

Antimicrobial activity of fermented products in baked bread

Microbiological quality of bread crust and crumbs is presented in Table 3. After baking, the total number of microorganisms in bread (experimental and control samples) was very low and varied from 14 to 210 CFU/g in the crust and from 33 to 405 CFU/g in the crumbs. Control and experimental bread were not contaminated with spores of aerobic mesophilic bacteria, and the contamination of all samples of bread with the spores of microscopic fungi was negligible.

The addition of *Silybum marianum* L. seeds fermented with *P. acidilactici* KTU05-7 had the greatest effect on the reduction of bacterial spoilage of bread in comparison with bread samples prepared without fermented products. Experimental bread crust and crumbs were less contaminated with spores of aerobic mesophilic bacteria and with fungi compared with the control sample. Differences in microbial contamination were noticed between bread crust and crumbs.

Table 3. Microbiological quality of bread crust and crumbs

Bread sampling location	Microbiological criteria	*t*(bread storage)/h	*N*(microorganisms)/(CFU/g)		
			control	with dried seeds	with fermented seeds
Crust	total number of microorganisms	2	190	210	14
		120	1100	570	60
	fungi	2	55	<10	<10
		120	390	320	110
	spore-forming aerobic mesophilic bacteria	2	<10	<10	<10
		120	<10	<10	<10
Crumbs	total number of microorganisms	2	33	405	67
		120	570	50	73
	fungi	2	45	<10	<10
		120	350	110	95
	spore-forming aerobic mesophilic bacteria	2	<10	<10	<10
		120	55	48	43

The application of fermented products resulted in a reduced microbiological spoilage of bread after 5 days of storage, and total number of microorganisms and fungal spores in the crust was 18.3 and 3.5 times lower, respectively, than in the control sample (without additives). The total number of microorganisms and fungal spores in the crumbs of the bread containing fermented *Silybum marianum* L. seeds after 5 days of storage was 7.8 and 3.7 times lower, respectively. Spores of aerobic mesophilic bacteria were found in the crumbs only after 5 days of bread storage and the additives of fermented products reduced their content (approx. 1.3 times).

This study showed that dried *Silybum marianum* L. seeds were also capable of decreasing the total number of microorganisms in crust after 5 days of storage by 1.9 times and of inhibiting fungal spores present in the crust as well as in crumbs (by 1.2 and 3.2 times, respectively).

These results are in agreement with other authors (*56,57*), who revealed that the use of microbial cultures in starter preparation increases the shelf life and safety of bakery products. This study highlights the possibility to use the tested LAB strains for fermentation of *Silybum marianum* L. seeds and production of wheat bread with improved microbiological characteristics.

The mean sensory ratings for each wheat bread sample are presented in Fig. 6. The thorough analysis of bread sensory attributes indicated that significant differences in odour and flavour of bread samples were noted when *Silybum marianum* L. seeds were added to the bread (Fig. 6a). Bread with fermented *Silybum marianum* L. seeds was commented to have a malty odour and flavour, and higher acidity, which is typical for sourdough bread. Darkness of the bread was directly related to increased fibre content. However, texture attributes as well as loaf

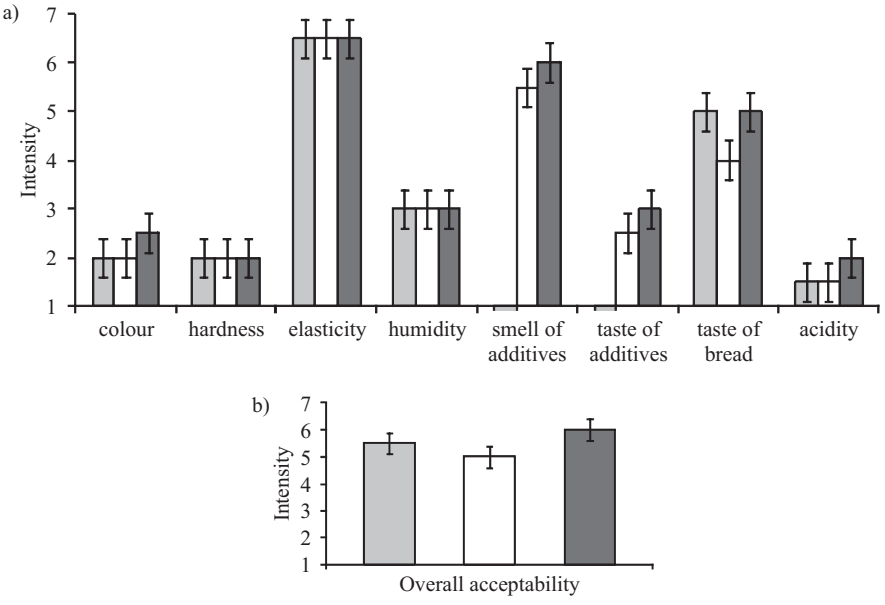

Fig. 6. The influence of the addition of *Silybum marianum* L. seeds on wheat bread sensory attributes and overall acceptability (□ control bread without additives, □ bread with 1 % dried *Silybum marianum* seeds, and ■ bread containing 2 % fermented *Silybum marianum* L. seeds)

volume were not found to be significantly different among the tested bread samples. The average scores of overall acceptability for wheat bread with added dried and fermented *Silybum marianum* L. seeds are presented in Fig. 6b. Data indicate that the bread with fermented *Silybum marianum* L. seeds was more palatable to consumers. This confirms that fermented *Silybum marianum* L. seeds have a great potential in food applications, especially in the development of functional foods including functional bakery products.

Conclusions

A pretreatment of plant seeds used as raw material by ultrasound reduced the total amount of pathogenic microorganisms, and the highest antimicrobial effect was achieved using solid-state fermentation of the treated seeds. After ultrasound pretreatment, the increased growth of LAB during fermentation was determined in comparison with the control samples. The solid-state fermentation showed positive changes of the phenolic content with the development of special flavour compounds. This study provides additional information and possibilities for the development of new effective tools for the elimination of bacterial contamination using a low temperature regime of plant material in SSF. The results obtained reveal that fermented *Silybum marianum* L. seeds are a suitable additive for natural flavouring of baked products.

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