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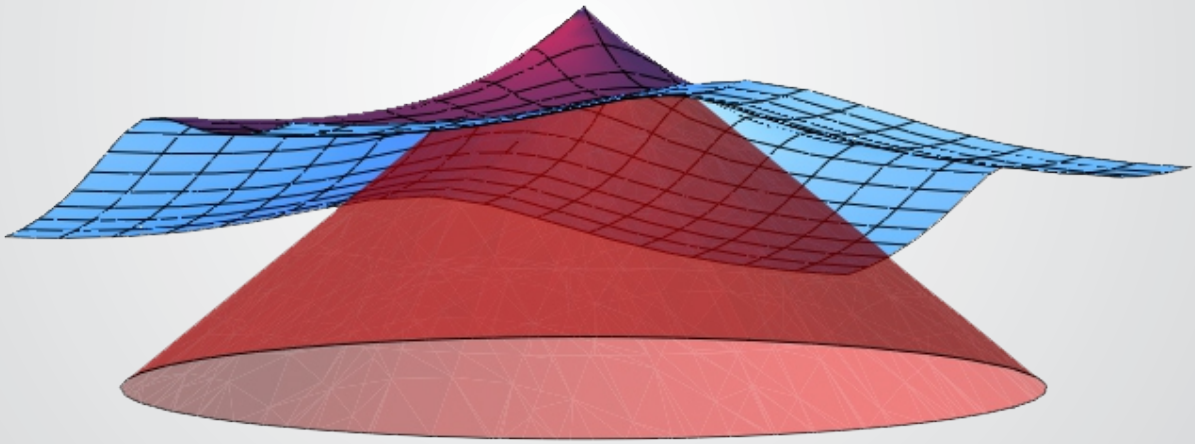

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CONTENTS

MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF SEVEN BACTERIAL STRAINS FOR WASTEWATER TREATMENT

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Abstract: *Seven bacterial strains are investigated. Six of them are isolated from soil and one – from starter cultures for raw dried meat products. It is found that all strains accumulate high concentrations of viable cells. They are similar in their inability to oxidize NH_4^+, to degrade haemoglobin and to be oxidaze – negative. Only bacterial strain Micrococcus 2 clearly expresses NO_3^- - reductase activity. Bacterial strains Bacteria 2 and Bacteria 8 exceed the rest in the expression of lipolytic activity. Bacterial strain Bacteria 4 exhibits the highest proteolytic activity. That is confirmed by the subsequent analysis of its enzyme profile using system API ZYM, the results of which show the strains' aminopeptidase, beta – galactosidase and glucosidase activities.*

Keywords: bacteria, morphological, physiological, biochemical methods, APIZYM, wastewater treatment

1. Introduction

Direct discharge of wastewater has a negative impact on receiving waters [4]. Proper sewage treatment limits the formation of ecological imbalance in aquatic ecosystems. Among sewage treatment systems biological methods are widely applied. They are based on the use of different types of bacteria. Among them are different species from the genera *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Micrococus*, *Lactobacillus*, *Pediococcus* [3, 6, 7, 9, 11, 12, 13, 14].

Special wastewater feature from food processing industry requires application of microorganisms with certain activities to utilize a variety of pollutants [5]. The aim of this work is analysis and identification of bacterial strains with expressed abilities for biological treatment of food processing wastewater.

2. Materials and methods

2.1. Microorganisms

Seven bacterial strains, marked as Bacteria 2, Bacteria 3, Bacteria 4, Bacteria 5, Bacteria 7, Bacteria 8, *Micrococcus* 2 are used in the present work. Bacterial strains Bacteria 2, Bacteria 3, Bacteria 4, Bacteria 5, Bacteria 7, Bacteria 8 are isolated from soil and bacterial strain *Micrococcus* 2 is isolated from starter cultures for raw dried meat products.

2.2. Nutrient media

***2.2.1. Malt extract medium (ME) with composition*:** malt extract (Kamenitza, Bulgaria) in 1:1 ratio with tap water (vol/vol). pH 6,5 – 7,0. The medium is sterilized for 25 minutes at 121ºC [2].

***2.2.2. Malt agar (MA) medium with composition*:** malt extract (Kamenitza, Bulgaria) in 1:1 ratio with tap water (vol/vol) + 2 % agar (w/vol). pH 6,5 – 7,0. The medium is sterilized for 25 minutes at 121º C [2].

***2.2.3. Luria – Bertany glucose medium (LBG) with composition (g/dm^3)*:** triptone (Difco) – 10 g, yeast extract – 5 g, NaCl – 10 g, glucose – 10 g. pH 7,5 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

***2.2.4. Luria – Bertany glucose agar medium (LBG agar) with composition (g/dm^3)*:** triptone (Difco) – 10 g, yeast extract – 5 g, NaCl – 10 g, glucose – 10 g, agar – 20 g. pH 7,5 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

***2.2.5. Soybean – caseine broth medium (SCB) with composition (g/dm^3)*:** triptone (Difco) – 17 g, soy peptone – 3 g, NaCl – 5 g, K_2HPO_4 – 2,5 g, glucose – 2,5 g. pH 7,3 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

***2.2.6. Soybean – caseine agar medium (SCB agar) with composition (g/dm^3)*:** triptone (Difco) – 15 g, soy peptone – 5 g, NaCl – 5 g, K_2HPO_4 – 2 g, glucose – 2,5 g, agar – 20 g. pH 7,3 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

***2.2.7. Medium for Micrococcus varians and Staphylococcus saprophyticus (SMS) with composition (g/dm^3)*:** yeast extract – 5 g, meat extract – 1 g, peptone – 10 g, NaCl – 5 g, glucose – 10 g, K_2HPO_4 – 0,5 g. pH 6,8 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

***2.2.8. Medium for Micrococcus varians and Staphylococcus saprophyticus (SMS agar) with composition (g/dm^3)*:** yeast extract – 5 g, meat extract – 1 g, peptone – 10 g, NaCl – 5 g, glucose (Scharlau) – 10 g, K_2HPO_4 – 0,5 g, agar – 20 g. pH

6,8 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

2.2.10. sition (g/dm^3): peptone – 10 g, meat extract – 5 g, yeast extract – 5 g, glucose – 20 g, K_2HPO_4 – 2 g, diammonium hydrogen cictrate – 2 g, $NaOOCCH_3$ – 5 g, $MgSO_4$ – 0,1 g, $MnSO_4$ – 0,05 g. pH 6,5 ± 0,2. The medium is sterilized for 25 minutes at 121º C.

2.2.11. Lactobacillus agar medium on the formulation of de Man, Rugosa and Sharpe (MRS agar) with composition (g/dm^3): peptone – 10 g, meat extract – 5 g, yeast extract – 5 g, glucose – 20 g, K_2HPO_4 – 2 g, diammonium hydrogen cictrate – 2 g, $NaOOCCH_3$ – 5 g, $MgSO_4$ – 0,1 g, $MnSO_4$ – 0,05 g, agar – 20 g. pH 6,5 ± 0,2. The medium is sterilized for 25 minutes at 121º C.

2.2.12. Citrate utilization medium (Simmons medium) with composition (g/dm^3): $Na(NH_4)_2PO_4$ – 1,5 g; KH_2PO_4 – 1 g; $MgSO_4.7H_2O$ – 0,2 g; Na – citrate – 3 g; alcohol solution of bromthimolblue – 1%; agar – agar – 2%. The medium is sterilized for 25 minutes at 121ºC [2].

2.2.13. Gelatinase activity medium with composition (g/dm^3): triptone (Difco) – 10 g; yeast extract (Scharlau) – 5 g; NaCl – 10 g; glucose (Scharlau) – 10 g; gelatine (DDR) – 250 g. pH 7,5 ± 0,2. Medium is dispenzed into tubes and is sterilized for 25 min at 121ºC [8].

2.2.14. Proteolytic activity medium with composition: malt agar medium with 10 % (vol/vol) solution additive (10 cm^3 milk/100 cm^3 water) of skimmed milk powder [2].

2.2.15. Lipolytic activity medium (Tween – 80 compounds hydrolisis) with composition (g/dm^3): peptone (Scharlau) – 10 g; NaCl – 5 g; $CaCl_2$ – 0,1 g; Tween – 80 (Merck) – 10 cm^3; agar – agar – 20 g. pH 7 – 7,4. The medium is sterilized for 25 minutes at 121ºC [2].

2.2.16. S – compound oxidation media.

2.2.16.1. Starckey broth medium with composition (g/dm^3): elemental S – 10 g; KH_2PO_4 – 3 g; $MgSO_4.7H_2O$ – 0,2 g; $CaCl_2.2H_2O$ – 0,2 g; $(NH_4)_2SO_4$ – 0,5 g; $FeSO_4$ – traces; indicator – Bromocresol purple. pH 8,0. The medium is prepared in two versions: with glucose (5 g/dm^3) and without glucose. It is sterilized at Koch apparatus for 30 min on three concecutive days [13].

2.2.16.2. NCL – broth medium with composition (g/dm^3): elemental S – 10 g; $(NH_4)_2SO_4$ – 0,2 g; $MgSO_4.7H_2O$ – 0,5 g; $CaCl_2.2H_2O$ – 0,25 g; $FeSO_4$ – traces; indicator – Bromocresol purple. The medium is prepared in two versions: with glucose (5 g/dm^3) and without glucose. It is sterilized at Koch apparatus for 30 min on three concecutive days [13].

2.2.9. Lactobacillus medium on the formulation of de Man, Rugosa and Sharpe (MRS) with compo

2.2.16.3. Thiosulphate agar medium with composition (g/dm^3): $Na_2S_2O_3$ – 5g; K_2HPO_4 – 0,1 g; $NaHCO_3$ – 0,2 g; NH_4Cl – 0,1 g; agar – agar – 20 g. pH 8,0. The medium is prepared in two versions: with glucose (5 g/dm^3) and without glucose. It is sterilized at Koch apparatus for 30 min on three concecutive days [13].

2.2.17. NO_3^- – reductase activity medium with composition (g/dm^3): peptone (Scharlau) – 5 g; meat extract (Scharlau) – 3 g; KNO_3 – 1 g. pH 7.0. The medium is sterilized for 25 minutes at 121ºC [3].

2.2.18. NH_4^+ – citrate medium (for nitrifying activity) with composition (mol/dm^3): Na – citrate.$2H_2O$ – $9{,}5.10^{-3}$; NH_4Cl – $9{,}35.10^{-3}$; KH_2PO_4 – $1{,}47.10^{-3}$; $MgSO_4.7H_2O$ – $1{,}62.10^{-4}$; $CaCl_2$ – $1{,}36.10^{-7}$; $FeSO_4.7H_2O$ as EDTA–complex – $3{,}6.10^{-5}$. The medium is sterilized for 25 minutes at 121ºC [10].

2.2.19. Blood agar (NCIPD, Bulgaria) with composition (g/dm^3): caseine hydrolisate – 14 g; NaCl – 5 g; peptone – 4,5 g; yeast extract – 4,5 g; defibrinated sheep blood – 70 cm^3; agar – agar – 12,5 g. pH 7,3 ± 0,2. All medium components are sterilized for 15 min at 121ºC. After cooling to 45ºC – 50ºC 70 cm^3 defibrinated sheep blood is added aseptically and the medium is poored in sterile Petri dishes [1].

2.2.20. Motility medium (NCIPD, Bulgaria) with composition (g/dm^3): peptone – 10 g, meat extract – 3 g, NaCl – 5 g, agar – agar – 4 g. pH 7,4 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

2.3. Cultivation and storage of the analysed microorganisms.

Bacterial strain Bacteria 2 is grown on LBG, bacterial strain Bacteria 3 is grown on MRS; bacterial strains Bacteria 4, Bacteria 5, Bacteria 7, Bacteria 8 are grown on SCB and bacterial strain *Micrococcus* 2 – on SMS at 30ºC in a thermostat for 48 h and are stored in a refrigerator at 4ºC for 2-3 weeks.

2.4. Analytical methods.

2.4.1. Morphological and cultural methods.

2.4.1.1. Gram staining determination. Determination of Gram staining of the bacterial strains is done by the method described in [2].

2.4.1.2. Examination of the spore formation ability. The ability of bacteria to form spores is examined. Using a flamed and cooled bacteriological loop a part in pre-developed on the respective nutrient medium 48-hour bacterial cultures is taken and a microscope slide preparation by Moller's method is done. Spores are rubine red and vegetative bodies – blue.

2.4.1.3. Determination of the number of viable cells in the development of the cultures in liquid medium. Sterile liquid media are inoculated under aseptic conditions with the respective bacterial suspension, 5 cm^3 medium is inoculated with 1 cm^3 of the inoculum of the studied cultures. After incubation in a thermostat at 30ºC for 48 h, the tenfold dilution method is performed and plates with the respective agar media are inoculated. Petri dishes are thermostatted at 30ºC for 48 h so that the strain would form single colonies, which are then counted.

2.4.2. Physiological and biochemical methods

2.4.2.1. Citrate utilization. Developed in containers with 30 cm^3 of the respective liquid media at 30ºC for 48 h bacterial strains are centrifuged at 3000 min^{-1} for 10 min. The supernatant is discarded and the biomass is resuspended in 5 cm^3 sterile physiological solution. The bacterial inoculum is taken using a sterile bacteriological loop and it is streak-spread on Petri dishes with Simmons medium. The inoculated plates are incubated at 30ºC for 48h. Positive results are recorded in the case of a colour change of the medium from green to blue.

2.4.2.2. Determination of the gelatinase activity of the tested strains.

The preparation method of the bacterial strains' inoculum is analogous to that in 2.4.2.10. The suspension of the tested strain is taken with a sterile bacteriological loop and is put in point onto a presterilized medium with gelatin. The tubes are incubated for 7 days at room temperature and the presence of crater melting is monitored [8].

2.4.2.3. Proteolytic activity determination using fusion agar method. The ability of bacterial strains to hydrolyze milk proteins in agar medium with milk additive is investigated. Wells with d = 6 mm are made on Petri dishes with medium for proteolytic activity. After inoculation, the plates are incubated at 30ºC for 48 hours. Results are reported as positive in the case of a formation of a brighter halo around the wells of the plates. A lack of halo is a sign of inability to utilize milk proteins.

2.4.2.4. Determination of the lipolytic activity using fusion agar method. Lipolytic activity includes the ability of the analyzed strains to hydrolyze the compounds of Tween - 80. Wells with d = 6 mm are made on Petri dishes with agar medium, described in 2.2.14. After inoculation the Petri dishes are cultivated at 30ºC for 1 - 7 days. Formation of a turbid zone around the wells, due to the precipitation of Ca - salts of the formed free fatty acids, indicates the presence of lipolytic activity.

2.4.2.5. Analysis of the ability of the studied strains to oxidize S-containing compounds. The analysis of the oxidation of S-containing compounds includes development of the bacterial strains on selective media. Tubes with sterile liquid Starkey medium and NCL medium "with" and "without" glucose are inoculated with 1 cm^3 bacterial suspension, prepared as described in 2.4.2.1.

The same suspension is used to streak plates with thiosulphate media "with" and "without" glucose. The plates and tubes are cultivated in a thermostat at 30ºC for 15 days. Changes in the colour of the indicator bromocresol purple (from purple to yellow on Starkey and thiosulphate media; and from yellow to dark yellow or purple on NCL – broth) are reported as positive results.

2.4.2.6. Determination of nitrifying activity of the studied strains. Nitrifying activity includes the development of the analyzed bacterial strains in a liquid medium with ammonium salts being the sole nitrogen source. A suspension of the tested bacterial strains is prepared, as described in 2.4.2.1.

1 cm^3 of it is used to inoculate tubes with 5 cm^3 of ammonia - acetate medium and the tubes are incubated at 30ºC for 24 hours. If the bacterial strain has nitrifying activity, NO_3^- iones would be formed in the medium. Their presence is determined by test - strips (110,020 Nitrate Test Merckoquant, Merck).

Some microorganisms have the ability to reduce NO_3^- to NO_2^-. Therefore, the content of NO_2^- in the medium is determined, using a test - strip (110,022 Nitrate Test Merckoquant, Merck). Results are recorded as positive in the presence of NO_3^- and NO_2^- or negative - when NO_3^- and NO_2^- are absent in the medium.

2.4.2.7. Determination of nitrate - reductase activity of the studied strains.

Prepared as in 2.4.2.1., the suspensions of the studied cultures are used to inoculate medium with KNO_3 - 1 cm^3 suspension is used to inoculate 5 cm^3 of sterile medium for the determination of nitrate - reductase activity. Incubation is carried out for 7 hours at 30ºC. The presence of NO_2^- in the medium is considered a positive result. Their presence is determined by a test – strip (110.022 Nitrate Test Merckoquant, Merck). Results are recorded as positive - in the presence of NO_2^- or negative - in the absence of NO_2^- in the medium.

2.4.2.8. Determination of hemolytic activity of the investigated cultures. Some microorganisms have the ability to utilize blood, breaking down red blood cells. Depending on the mechanism of their hydrolysis there are three types of hemolytic activity. In α - hemolysis iron from hemoglobin is oxidized and the colonies become dark - green. In β - hemolysis the erythrocytes and the hemoglobin in them are degraded, and a bright halo is formed around some

of the colonies. γ – hemolysis is observed in the case of no hemoglobin hydrolysis.

Using a bacteriological loop inoculum is taken from the suspension of the tested bacterial strain, prepared as in 2.4.2.1. and it is stroked on blood agar. The inoculated plates are cultured for 48 hours at 30ºC. The presence of hemolytic activity is recorded: in the cases of α – or β – hemolysis, the result is positive, while for γ – hemolysis, it is negative.

2.4.2.9. Determination of catalase activity of the analysed cultures. Catalase activity is determined by the method described in [13].

2.4.2.10. Determination of oxidase activity of the investigated cultures.

The oxidase activity test includes analysis of the suspension of the studied strains for the presence of the enzyme cytochrome - oxidase. In the presence of molecular oxygen, cytochrome - oxidase can reduce the number of organic substances, including the reagent NaDi (1 - naphthol + diamine dimetilparaphenylene) with the formation of indophenole blue. Test - strips for oxidase activity (Microbiology Bactident Oxidase 1.13300.0001, Merck) are pipetted with the suspension of bacterial strains prepared as in 2.4.2.1.

After 20-60 s the result is compared to a colour scale. Positive result are recorded in the case of a color change of the strip from white to blue to blue - violet.

2.4.2.11. Determination of the profile of enzyme activity of the studied cultures.

The determination of the profile of enzyme activity is performed, using the test kit API ZYM (BioMerieux) for semiquantitative determination of the enzyme profile of the studied strain. Fresh 24-hour culture of the tested strain is centrifuged for 15 minutes at 5000 g, the obtained biomass precipitate is washed twice and resuspended in API suspension medium. The API ZYM strips are placed in the incubation boxes and the microtubes are inoculated with the prepared cell suspension. The sample is incubated for 4 to 4,5 hours at 30°C. After the incubation one drop of reagent A and one drop of reagent B are pipetted into each microtubule. After 5 min staining result is reported according to the colour scheme described in the manufacturer's instructions. The enzyme activity is determined according to the colour scale from 0 (no enzyme activity) to 5 (maximum enzyme activity).

3. Results and discussion

The ability of the bacterial strains to grow in five cultural media is investigated. The results represented in Table 1 show that the best liquid medium for the bacterial strains' development is respectively: LBG for Bacteria 2; MRS for Bacteria 3; SCB for Bacteria 4, Bacteria 5, Bacteria 7, Bacteria 8 and SMS for *Micrococcus* 2.

Table 1. *Media for the development of the bacterial strains*

Strain	Medium				
	LBG	**ME**	**SCB**	**MRS**	**SMS**
Bacteria 2	Abundant sludge, Uniform turbidity	–	–	–	–
Bacteria 3	Turbidity	–	–	Abundant sludge with a column of enlightened zone	–
Bacteria 4	–	–	Abundant sludge, Uniform turbidity	–	–
Bacteria 5	–	–	Abundant sludge, Uniform turbidity	–	–
Bacteria 7	–	–	Abundant sludge, Uniform turbidity	–	–
Bacteria 8	–	–	Abundant sludge, Uniform turbidity	–	–
Micrococcus 2	–	–	–	–	No turbidity, grainy sludge

The amount of viable cells is determined during cultivation of the investigated bacterial strains on the respective liquid medium. The test results (Table 2) show that all bacterial strains accumulate high concentrations of viable cells (over 10^{11} cfu/cm^3) for 48 h cultivation at 30°C.

When examining the ability of the isolated bacterial strains to form spores and the Gram staining it is found that they are all Gram (+) and don't form spores (Table 3).

Table 2. *Concentration of viable bacterial cells, cultivated in the respective liquid medium*

Bacterial strains	Average (cfu/cm^3)
Bacteria 2	$8{,}0.10^{11}$
Bacteria 3	$3{,}0.10^{11}$
Bacteria 4	$5{,}9.10^{12}$
Bacteria 5	$7{,}0.10^{11}$
Bacteria 7	$4{,}2.10^{12}$
Bacteria 8	$2{,}2.10^{12}$
Micrococcus 2	$5{,}4.10^{12}$

Table 3. *Gram staining and spore forming ability of the investigated bacterial strains*

Bacterial strains	Gram staining	Spore formation
Bacteria 2	Gram (+)	–
Bacteria 3	Gram (+)	–
Bacteria 4	Gram (+)	–
Bacteria 5	Gram (+)	–
Bacteria 7	Gram (+)	–
Bacteria 8	Gram (+)	–
Micrococcus 2	Gram (+)	–

The experimental results in Table 4 show significant similarities between investigated bacterial strains. They don't hydrolyse gelatin (except bacterial strain Bacteria 3), don't oxidize NH_4^+, don't utilize citrate, they are non-motile, don't degrade heamoglobin (γ – haemolysis) and are oxidoreductase – negative. All tested bacterial strains don't utilize sulfur compounds at pH < 5 (Starkey medium) and at pH 8 (media $Na_2S_2O_3$ and NCL). Their inability to grow in sulfur containing media without glucose defines them as heterotrophic microorganisms. In the presence of glucose grow bacterial strains Bacteria 7, Bacteria 8, *Micrococcus* 2, but only strain Bacteria 7 reduces sulfur compounds from the $Na_2S_2O_3$ medium. Bacterial strains Bacteria 2, Bacteria 3, Bacteria 8 and *Micrococcus* 2 are able to utilize sulfur compounds on NCL medium with glucose.

All strains, except Bacteria 4 and Bacteria 5, exhibit lypolytic activity, which is most important for Bacteria 2 and Bacteria 8, followed by *Micrococcus* 2, Bacteria 7 and Bacteria 3.

The investigated microorganisms are distinguished by the catalase activity, bacterial strains Bacteria 2, Bacteria 3 and Bacteria 7 are catalase – negative, while Bacteria 4, Bacteria 5, Bacteria 8 and *Micrococcus* 2 – catalase – positive. Only bacterial strain *Micrococcus* 2 exhibits nitrate – reductase activity.

All investigated bacterial strains have proteolytic activity, which is highest in Bacteria 4, and lowest – in Bacteria 5.

As a result of these experimental studies for further work are selected the following bacterial strains: Bacteria 2, Bacteria 4, *Micrococcus* 2. Aiming at more complete analysis of these bacterial strains' application ablilities in the field of wastewater treatment from food processing industry their enzyme profile is investigated with the system API ZYM. Experimental results are presented in Table 5.

Bacterial strain Bacteria 2 has leucine-aminopeptidase, valine-aminopeptidase, β-galactosidase, naphthol – AS – BL – phosphohydrolase, α-galactosidase, α- and β-glucosidase, alkaline phosphatase, lipase C_4, lipase C_8, acid phosphatase, cysteine-aminopeptidase, β-glucuronidase and α-glucoseaminidase activities. The big set of lipolytic and proteolytic enzymes, which are included in the system API ZYM proves relevant activities as the ones described for this strain in Table 4.

Of all investigated strains Bacteria 4 shows the strongest ability to degrade milk proteins (Table 4). The reason probably lies in the enzymes of the strain – leucine-aminopeptidase, valine-aminopeptidase, cysteine-aminopeptidase. In this culture the possible reason for the absence of lipolytic activity (Table 4) is the little content of lipolytic enzymes – lipase C_8, acid phosphatase, naphthol – AS – BL – phosphohydrolase. This strain can utilize different carbohydrates with α-galactoside, β-galactoside, α-glucoside, β-glucoside, α-glucoseamide bonds. Of all bacterial strains, tested with API ZYM only Bacteria 4 expresses α-fucosidase activity.

Micrococcus 2 shows only lipolytic enzyme activities – alkaline phosphatase, lipase C_4, lipase C_8, acid phosphatase, naphthol – AS – BL – phosphohydrolase. This confirms the results obtained for the lipase activity, but not those for the proteolytic activity (Table 4).

Table 4. *Biochemical features of the analyzed bacterial strains*

Strain			**Bacteria 2**	**Bacteria 3**	**Bacteria 4**	**Bacteria 5**	**Bacteria 7**	**Bacteria 8**	***Micrococcus* 2**
Lipolytic activity, mm			+ 14,5±0,5	+ 8,0±0,0	–	–	+ 10,5±0,5	+ 14,2±1,2	+ 11,3±0,8
Nitrifying activity			–	–	–	–	–	–	–
Gelatinase activity			– anaerobes	+ anaerobes	– anaerobes	– facultative anaerobes	– facultative anaerobes	– facultative anaerobes	– facultative anaerobes
Oxidation of S – containing compounds	Media with glucose	$Na_2S_2O_3$	–	–	–	–	+ Growth	– Growth	– Growth
		Starkey	–	–	–	–	–	–	–
		NCL	–	–	–	–	–	+	+
	Media without glucose	$Na_2S_2O_3$	+	+	–	–	–	+	+
		Starkey	–	–	–	–	–	–	–
		NCL	–	–	–	–	–	–	–
Catalase activity			–	–	+	+	–	+	+
Oxidoreductase actividy			–	–	–	–	–	–	–
NO_3^- - reductase activity			–	–	–	–	–	–	+
Proteolytic activity	24 h		+ 13,2±1,2	+ 16,3±2,4	+ 19,8±0,4	–	+ 16,7±0,5	+ 16,5±1,25	+ 14,3±1,25
	48 h		+ 25±1,2	+ 25,8±2,1	+ 29,7±3,3	+ 10,5±2,1	+ 27,5±1,3	+ 25,2±0,4	+ 22±2,0
Citrate utilization			–	–	–	–	–	–	–
Hemolytic activity			γ	γ	γ	γ	γ	γ	γ
Motility			–	–	–	–	–	–	–

Table 5. *Enzyme profile of strains Bacteria 2, Bacteria 4 and Micrococcus 2 using the system API ZYM*

№	**Enzyme**	**Strain**					
		Bacteria 2		**Bacteria 4**		***Micrococcus* 2**	
1	Control		–		–		–
2	Alkaline phosphatase		1		–		1
3	Lipase C_4		1		–		1
4	Lipase C_8		2		1		2
5	Lipase C_{14}		–		–		–
6	Leucine-aminopeptidase		5		5		–
7	Valine-aminopeptidase		5		4		–
8	Cysteine-aminopeptidase		1		2		–
9	Trypsin		–		–		–
10	Chymotrypsin		–		–		–
11	Acid phosphatase		2		2		2
12	Naphthol – AS – BL – phosphohydrolase		3		2		2
13	α-galactosidase		3		2		–
14	β-galactosidase		5		5		–
15	β-glucuronidase		1		–		–
16	α-glucosidase		3		4		–
17	β-glucosidase		3		5		–
18	α-glucoseaminidase		1		2		–
19	α-manosidase		–		–		–
20	α-fucosidase		–		1		–

As a result of the conducted experimental analyses the following more important conclusions can be formulated:

1. A morphological, physiological and biochemical characterization of six bacterial strains, isolated

from soil and one bacterial strain, isolated from starter cultures for raw dried meat products is done.

2. The enzyme profile of bacterial strains Bacteria 2, Bacteria 4 and *Micrococcus* 2 is determined, thus giving possibilities for their combination and application in wastewater treatment in the food industry.

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MORPHOLOGICAL CHARACTERISTICS AND GENETIC IDENTIFICATION OF SEVEN BACTERIAL STRAINS FOR WASTEWATER TREATMENT

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Abstract: *Seven bacterial strains were investigated. Five of them were isolated from soil and one – from starter cultures for raw dried meat products. It was found that all bacterial strains are similar in their colonial characteristics. In cell description four of them are short rods, and two – bacterial strains Bacteria 7 and Bacteria 8 are very short rods. Only bacterial strain Micrococcus 2 is coccus. Genetic identification of the 16S rRNA gene show that bacterial strains belong to the genera Lactobacillus and Staphylococcus.*

Keywords: bacteria, morphological, genetic identificatiom, *Lactobacillus casei, Lactobacillus plantarum, Staphylococcus hominis*

Introduction

Wide occurrence of bacteria and their great adaptability to unfavorable conditions allow their application in areas not typical for them, such as biological wastewater treatment. The most common bacteria used in wastewater treatment processes include the genera *Pseudomonas*, *Bacillus*, *Micrococcus*, *Lactobacillus* [1, 3, 6, 8, 9, 11,12].

Proper identification gives the capability to compare novel bacterial strains with already investigated strains and to research new special features among them [5].

The aim of this study was to identify bacterial species isolated from soil and from starter cultures for raw dried meat products for future application in sewage treatment processes from food processing industry.

Materials and methods

1.1. Microorganisms

In this work are used seven bacterial strains, marked as Bacteria 2, Bacteria 3, Bacteria 4, Bacteria 5, Bacteria 7, Bacteria 8, *Micrococcus* 2. Bacterial strains Bacteria 2, Bacteria 3, Bacteria 4, Bacteria 5, Bacteria 7, Bacteria 8 were isolated from soil and bacterial strain *Micrococcus* 2 was isolated from starter cultures for raw dried meat products.

Lactobacillus casei subsp. casei ATTC 393, *Lactobacillus plantarum* NRRL B-14768, *Staphylococcus hominis subsp. novobiosepticus* GTC 1228 have been used as reference culture strains for the genetic sequence analysis.

1.2. Nutrient mediums

1.2.1. Luria – Bertany glucose medium (LBG) with composition (g/dm^3): triptone (Difco) – 10 g, yeast extract – 5 g, NaCl – 10 g, glucose – 10 g. pH 7,5 ± 0,2. The medium is sterilized for 25 minutes at 121ºC [2].

1.2.2. Luria – Bertany glucose agar medium (LBG agar) with composition (g/dm^3): triptone (Difco) – 10 g, yeast extract – 5 g, NaCl – 10 g, glucose – 10 g, agar – 20 g. pH 7,5 ± 0,2. The medium is sterilized for 25 minutes at 121ºC [2].

1.2.3. Soybean – caseine broth medium (SCB) with composition (g/dm^3): triptone (Difco) – 17 g, soy peptone – 3 g, NaCl – 5 g, K_2HPO_4 – 2,5 g, glucose – 2,5 g. pH 7,3 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

1.2.4. Soybean – caseine agar medium (SCB agar) with composition (g/dm^3): triptone (Difco) – 15 g, soy peptone – 5 g, NaCl – 5 g, K_2HPO_4 – 2 g, glucose – 2,5 g, agar – 20 g. pH 7,3 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

1.2.5. Medium for Micrococcus varians and Staphylococcus saprophyticus (SMS) with composition (g/dm^3): yeast extract – 5 g, meat extract – 1 g, peptone – 10 g, NaCl – 5 g, glucose – 10 g, K_2HPO_4 – 0,5 g. pH 6,8 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

1.2.6. Medium for Micrococcus varians and Staphylococcus saprophyticus (SMS agar) with composition (g/dm^3): yeast extract – 5 g, meat extract – 1 g, peptone – 10 g, NaCl – 5 g, glucose

(Scharlau) – 10 g, K_2HPO_4 – 0,5 g, agar – 20 g. pH 6,8 ± 0,2. The medium is sterilized for 25 minutes at 121ºC.

1.2.7. Lactobacillus medium on the formulation of de Man, Rugosa and Sharpe (MRS) with composition (g/dm^3): peptone – 10 g, meat extract – 5 g, yeast extract – 5 g, glucose – 20 g, K_2HPO_4 – 2 g, diammonium hydrogen cictrate – 2 g, CH_3OOCNa – 5 g, $MgSO_4$ – 0,1 g, $MnSO_4$ – 0,05 g. pH 6,5 ± 0,2. The medium is sterilized for 25 minutes at 121º C [2].

1.2.8. Lactobacillus agar medium on the formulation of de Man, Rugosa and Sharpe (MRS agar) with composition (g/dm^3): peptone – 10 g, meat extract – 5 g, yeast extract – 5 g, glucose – 20 g, K_2HPO_4 – 2 g, diammonium hydrogen cictrate – 2 g, CH_3OOCNa – 5 g, $MgSO_4$ – 0,1 g, $MnSO_4$ – 0,05 g, agar – 20 g. pH 6,5 ± 0,2. The medium is sterilized for 25 minutes at 121º C [2].

1.3. Cultivation and storage of the analysed microorganisms.

Bacterial strains Bacteria 2 is grown on LBG, Bacteria 3 are grown on MRS; bacterial strains Bacteria 4, Bacteria 5, Bacteria 7, Bacteria 8 are grown on SCB and bacterial strain *Micrococcus* 2 – on SMS at 30ºC in a thermostat for 48 h and are stored in a refrigerator at 4ºC for 2-3 weeks.

1.4. Analytical methods.

1.4.1. Morphological methods.

1.4.1.1. Cellular and colonial morphology.

Description of cellular and colonial morphology of the studied bacterial strains is performed by microscopic observation of a smear on a slide of single colonies after their development on the respective for each bacterial strain agar medium [7].

1.5. Genetical methods;

1.5.1. Total DNA isolation;

Preliminary developed at 30°C for 48 h bacterial strains are ce centrifuged for 10 min at 3000 min^{-1}. Supernatant is discarded and the biomass is washed twice with sterile physiological solution. To the biomass of each culture 600 µl lysis buffer [composition 550 µl TE buffer + 50 µl lysozyme (25 mg/ml) and RNA-ase (20 mg/ml)] is added and the reaction is incubated for 1 h at 37ºC. After that 45 µl SDS solution (0,5 %) and 5 µl proteinase (20 mg/ml) are added, which is followed by incubation for 1,5 h at 65ºC. After that 100 µl solution NaCl (0,5 %) is added and the treated sample is stirred using Vortex for 20 s. Then 80 µl pre-tempered 65ºC CTAB is added and after 10 min each sample is homogenized to milky color. After incubation for 10 min at 65ºC the samples are incubated for 5 min at room temperature then an equal volume phenol with pH 7,5 is added, then is homogenized and centrifuged for 5 min at 13 000 min^{-1}. The aqueous layer from the supernatant is collected in new tube without shaking. An equal volume of phenol:[$CHCl_3$:isoamyl alcohol 24:1] 1:1 is added and homogenized using Vortex for 1 min. The resulting mixture is centrifuged for 5 min at 13 000 min^{-1}. The aqueous layer from the supernatant is collected in new tube without shaking. An equal volume of $CHCl_3$, is added and homogenized using Vortex for 1 min. The resulting mixture is centrifuged for 5 min at 13 000 min^{-1}. The aqueous layer from the supernatant is collected in new tube without shaking. 2,5 volumes of cold C_2H_5OH (96 %) are added and incubated for 12 h at -20ºC. The samples are centrifuged for 15 min at 13 000 min^{-1}, the ethanol is discarded, the precipitate is washed with 1 ml C_2H_5OH (75 %) and is centrifuged for 15 min at 13 000 min^{-1}. The ethanol is decanted and sam[les are drained on paper. The samples are dried at 65°C for 15-20 min, after which according to the volume of precipitate 50, 150 or 250 µl distilled water is added to each sample, followed by thorough homogenization. The purity of the isolated DNAs is determined by measurement the absorption on a spectrophotometer and the ratio A_{260}/A_{280} for each sample is calculated.

1.5.2. PCR reactions, visualisation and purification of DNA fragments.

All PCR reactions are performed using VWR kit for PCR (VWR taq2xmastermix, 2mM $MgCl_2$) in a final volume of 60 µl in Progene cycler (Techne, UK). In amplification of the isolated DNA universal primers for the gene of 16S rRNA of the investigated bacterial strains – 27F и 1492R (s) are used [4].

To 2 µl of each of the isolated DNA 30 VWR kit for PCR (VWR taq2xmastermix, 2mM $MgCl_2$), 2 µl 16S rDNA primers (Novozymes), 26 µl distilled water. Amplification program includes: denaturation – 95°C for 3 min, 35 cycles - 94° C for 30 s, 53° C for 30 s, 72°C for 2 min, final elongation – 72°C for 7 min.

After completion of the PCR – reaction the obtained products are visualized by gel-electrophoresis. 55 µl of the resulting PCR product at amplification of investigated strains' 16S rDNA is mixed with 9 µl solution [sucrose (40 %) + bromthymol blue], and then separated in a 2 % agarose gel on gel-electrophoresis at 100 V on BioRad sub-cell GT (Japan). The concentration of desired DNA – fragments from PCR – reaction is determined by comparison with the marker DNA – molecular weight marker III. After visualization with ethidium bromide solution (0,5 µg/ml) on UVP Documentation System (UK) for 30 min the DNA – fragments are purified with Illustra^{TM} GFX^{TM} PCR and Gel Band Purification Kit (General Electric Company, UK).

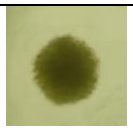
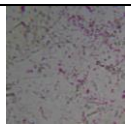
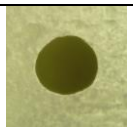
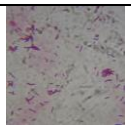
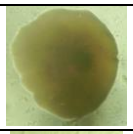
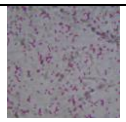
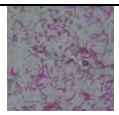
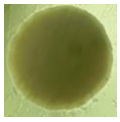
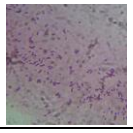
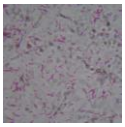
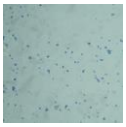
The desired DNA – fragments of the respective bacterial strain are excised and transferred in a DNase – free 1,5 ml microcentrifuge tube, where are mixed with Capture buffer (10 µl Capture buffer type 3 for each 10 mg agarose gel slice) by inversion and are incubated at 60ºC. After the complete agarose dissollution the mixture is homogenized using Vortex and centrifuged for 1 min at 13 000 min^{-1}. 600 µl of the Capture buffer type 3-sample mix is transferred to assembled GFX MicroSpin column and Collection tube. After 1 min at room temperature for column hydration the assembled complex is centrifuged for 50 sec at 13 000 min^{-1}. The liquid mixture in the Collection tube is discarded. The sample binding step is repeated until all samples are loaded in the GFX MicroSpin column. 500 µl Wash buffer type 1 is added following by centrifugation for 1 min at 13 000 min^{-1}. The Collection tube is discarded and GFX MicroSpin column is assembled to DNase – free 1,5 ml microcentrifuge tube. 50 µl Elution buffer type 4 or type 6 are added to the column. After 1 min at room temperature for column hydration the assembled complex is centrifuged for 1 min at 13 000 min^{-1}. The resulting purified rDNA fragments are stored at – 20ºC.

The purified DNA – fragments are sequenced by Macrogen Inc., The Netherlands according to the method of Sanger [10]. The genetic sequences of the investigated bacterial strains are preceded with CLC Sequence Viewer 6 and the online database of the Basic Local Alignment Search Tool (BLAST).

Results and discussion

The colonial characteristics of the researched strains are determined after inoculating the respective for each bacterial strain agar medium and cultivation for 48 h (Table 1).

Table 1. *Colonial characteristics and cell morphology of the investigated bacterial strains*

Strain	**Colonial characteristics**		**Cell morphology**	
	Colony description	**Visualisation**	**Cell description**	**Visualisation**
Bacteria 2	Round colonies with wave like ends, protuberanted, white to whitish, 2-3 mm in diameter		Short rods with rounded ends, organized one by one, by couples and in short chains	
Bacteria 3	Round colonies with wave like ends, protuberanted, white to whitish, 2-3 mm in diameter		Short rods with rounded ends, organized one by one, don't form short chains	
Bacteria 4	Round colonies with equal ends, protuberanted, white to whitish, 2-3 mm in diameter		Short rods with rounded ends, almost like cocci. Organized one by one, rarely in short chains	
Bacteria 5	Round colonies with wave like ends, protuberanted, white to whitish, 2-3 mm in diameter		Short rods with rounded ends, organized one by one, by couples and in short and long chains	
Bacteria 7	Round colonies with snowflake like ends, protuberanted, white to whitish, thready with inequal surface, 2-3 mm in diameter		Very short rods, thickened, with round ends and organized in chains	
Bacteria 8	Round colonies with snowflake like ends, protuberanted, white to whitish, thready with inequal surface, 2-3 mm in diameter		Fine short rods with rounded ends, organized one by one, by couples and in short chains	
Micrococcus 2	Round colonies with wave like ends, protuberanted, whitish, 2-3 mm in diameter		Cocci, according to spatial situation can be refered to *Micrococcus* genius. Organized one by one,rarely in groups	

Cell morphology and spore formation is determined by coloured microscope preparations. All bacterial strains are similar in their colonial appearance. Four of them (Bacteria 2, Bacteria 3, Bacteria 4, Bacteria 5) are short rods, and two of them – (bacterial strains Bacteria 7 and Bacteria 8) – are very short

rods. From all rods only the bacterial strain Bacteria 3 doesn't form chains.

Only the bacterial strain *Micrococcus* 2 is coccus.

The genetic identification of investigated bacterial strains has been done through 16S rRNA gene sequencing. The results from the sequencing are shown in Table 2. Bacterial strains Bacteria 2 (Figure 1), Bacteria 5, Bacteria 7, Bacteria 8 and Bacteria 3 are identified as *Lactobacillus casei*. Bacterial strain Bacteria 4 (Figure 2) belong to the species *Lactobacillus plantarum* ssp. *plantarum*, while *Micrococus* 2 (Figure 3) – to species *Staphylococcus hominis*.

Table 2. *Genetic identification with DNA-sequencing of 16S rRNA gene of investigated bacterial strains*

Strain	**Species**	**Reliability, %**
Bacteria 2	*Lactobacillus casei* ssp. *casei* 2	99
Bacteria 3	*Lactobacillus casei* ssp. *casei* 3	99
Bacteria 4	*Lactobacillus plantarum* ssp. *plantarum*	99
Bacteria 5	*Lactobacillus casei* ssp. *casei* 5	99
Bacteria 7	*Lactobacillus casei* ssp. *casei* 7	96
Bacteria 8	*Lactobacillus casei* ssp. *casei* 8	99
Micrococus 2	*Staphylococcus hominis* 2	99

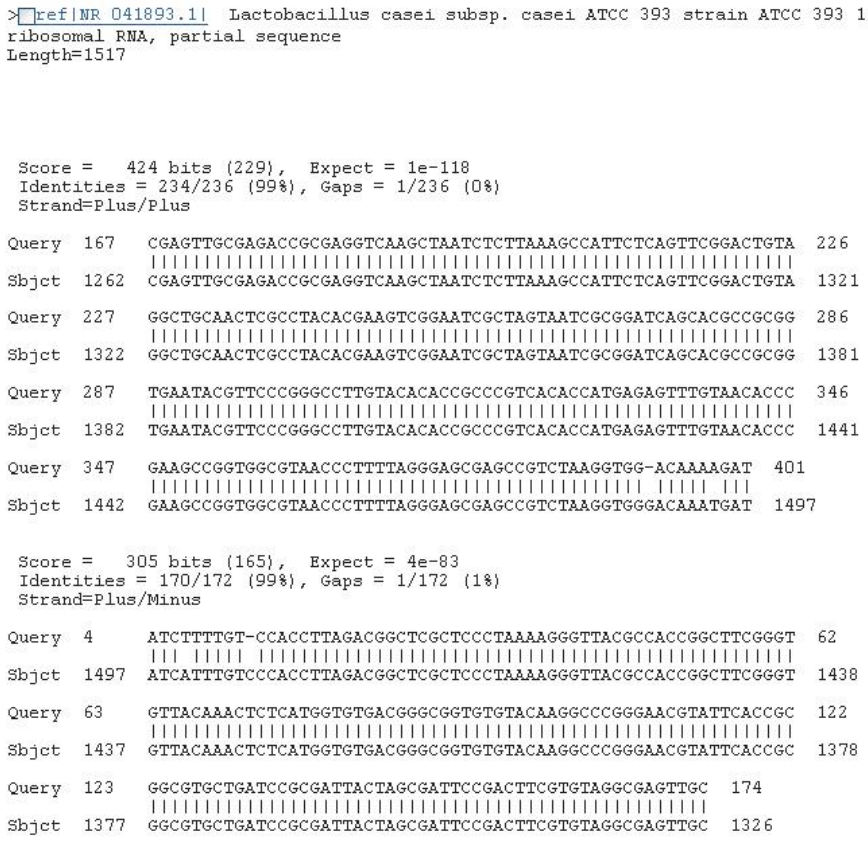


Figure 1. *Comparison of the nucleotide sequences of 16S rRNA gene of bacterial strain Bacteria 2 and partial sequence of the 16S rRNA gene of Lactobacillus casei ssp. casei ATTC 393;*

As a result of conducted experiments the following conclusions can be formulated:

1. The colonial and cellular characteristics of the bacterial strains isolated from different sources have been performed.

2. Using the molecular – genetic methods for identification (sequencing of the 16S rDNA) the species' identity of the seven bacterial strains have been determined.

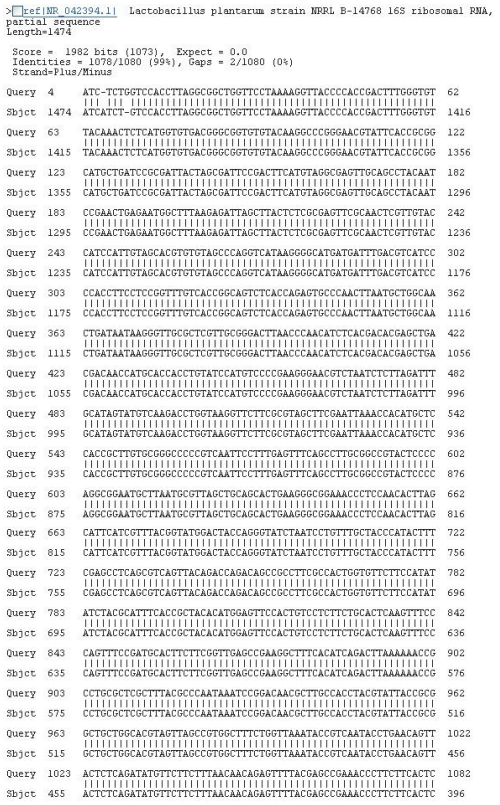


Figure 2. *Comparison of the nucleotide sequences of the 16S rRNA gene of the bacterial strain Bacteria 4 and the partial sequence of the 16S rRNA gene of Lactobacillus plantarum NRRL B-14768.*

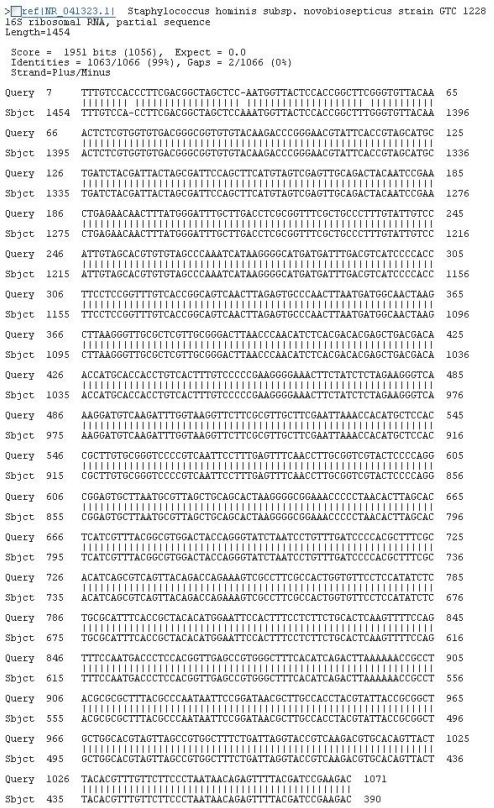


Figure 3. *Comparison of the nucleotide sequences of 16S rRNA gene of 16S rRNA gene of bacterial strain Micrococcus 2 and partial sequence of 16S rRNA gene of bacterial strain Staphylococcus hominis ssp. novobiosepticus GTC 1228;*

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PHYSICOCHEMICAL AND MICROBIOLOGICAL CHARACTERISTICS OF SPRING WATERS IN HASKOVO

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Abstract. *The physicochemical properties of the spring waters in the region of Haskovo are determined. It is shown that when 18 control parameters included in the study are considered spring water from Yabalka village, "Osmanova cheshma" and spring water from Dobrich meet the requirements for drinking water.*

The spring waters from six water sources are characterized by microbiological indicators, the pathogenic microorganisms are determined using the membrane method. It was found that non-thermal spring water from the village of Mineralni bani and spring water from the village of Radievo meet the standard requirements.

Keywords: spring water, drinking water, physicochemical, microbiological indicators

I. Introduction

Bulgaria is one of the richest countries in mineral springs in Europe. It ranks second after Iceland. The total number of mineral springs in Bulgaria is about 225. On the basis of their position certain characteristics are monitored. The springs located north of the Balkan Mountains, are with lower temperatures and are easily achieved by probes. Their total number is almost two times smaller than that of the springs located south of the Balkan Mountains. About 148 springs in South Bulgaria are known. The springs of natural origin and with higher water temperature are predominant. The reasons for this lie in the combination of hydrological features and ongoing tectonic processes in the Earth's crust [Ignatov et al., 2012]. Springs can be divided into cold, warm and hot springs depending on their nature. The first group includes those having a temperature up to 37°C, the second one consists of those with a temperature range between 37°C and 60°C, and the third group includes the springs with temperature over 60°C. The hottest spring in Bulgaria is the Separeva banya spring with temperature of 101.4°C.

Spring waters have different mineralogical characterization. Their composition is determined by the composition of the rocks through which the water has passed and the solubility of the minerals in them [Ignatov et al., 2012]. There are different types of water in Bulgaria. Some of them are suitable for everyday use, while others can lead to complications if used excessively.

Mineral waters are natural waters from a natural spring or a borehole that have an impact on the human body due to the elevated content of chemical components or the higher temperature. Mineral waters in Bulgaria are valuable because of their diverse chemical composition and mineralization, the type of salts dissolved in them, the curative gases and the biologically active trace elements. All possible types of mineral waters that exist in nature and have different characteristics can be found in our country [Mosin et al., 2012]. Some of them are hydro (Hisar, Pavel banya, Birimirtsi, Kniajevo, Targovishte), others are sulfate (Momin prohod, Haskovski mineralni bani, Pchelin, Dolna banya, Bata banya), still others are chloride (Mirovo, Dolen chiflik, Shabla), while others are fluorine (Pavel banya, Separeva banya, Ognianovo), the fifth group includes those that are radioactive and radon (Momin prohod, Narechen, Strelcha, Hisar, Kostenec, Pchelin, Dolna banya, Shipkovski bani), the sixth group consists of those that are carbon-acid (Mihalkovo, St. Karadjovo), the seventh group is the group of the hydrogen sulfide ones (Separeva banya, Kyustendil, Shabla, Birimirtsi), and the eighth group is mixed (Bankya, Gorna banya, Varshetz, Sofia, Burgaski mineralni bani, Velingrad, Merichleri). Quite a number of mineral waters are also of the group of the specific-silicon waters (Burgaski mineralni bani, Belen, Blagoevgrad, Varvara, Velingrad). This enormous and valuable to our country

treasure draws perspectives for lively tourist connections and routes.

II. Materials and Methods

The healing and spring waters in Haskovo region used in the present study are from the following springs: healing spring in the village of Mineralni bani; thermal healing spring in Mineralni bani with water temperature 57°C; spring around the village of Gorski izvor; spring around the village of Yabalkovo, a spring around the village of Dobrich, a spring around the village Radievo.

Media

Meat peptone agar (MPA). Composition (in %) - meat water, peptone - 1%; agar - agar - 2%.

Endo medium (for determination of *Escherichia coli* and coliform bacteria). Composition (g/dm^3): peptone - 5.0; tryptone - 5.0; lactose – 10.0; Na_2SO_3 – 1.4; K_2HPO_4 – 3.0; magenta - 0.14; agar - agar - 12.0. pH is adjusted to 7.5 - 7.7.

Meat peptone gelatin (MPG) (for identification of *Pseudomonas aeruginosa*). Composition: (in %) – Meat peptone broth; gelatin – 25%, pH is adjusted to 7.0 – 7.2.

Medium for enterococci (esculin - bile agar).

Medium for sulfate reducing bacteria (Iron Sulfite Modified Agar).

Wilson - Bleer medium (for determination of sulfate reducing sporeforming anaerobes (*Clostridium perfringens*). Composition (g/dm^3): MPA – 30; 20% solution Na_2SO_3 – 100 cm^3; 20% glucose solution - 50 cm^3; 8% solution Fe_2SO_4 - 10 cm^3.

Methods for physical and chemical analysis

Method for determination of the colour according to the Rublyovska Scale - method BS 8451: 1977 [1];

Method for determination of the odor at 20°C - method BS 8451: 1977, technical means - Mercury thermometer, conditions № 21 [1];

Method for determination of turbidity - EN ISO 7027, technical means - turbidity meter type TURB 355 IR ID № 200807088 [2];

Method for determination of pH - BS 3424: 1981, technical means - pH meter type UB10 ID № UB10128148 [3];

Method for determination of oxidation - BS 3413: 1981 [4];

Method for determination of chlorides - BS 3414: 1980 [5];

Method for determination of nitrates - VLM - NO_3 - №2, technical means – photometer "NOVA 60 A" ID № 08450505 [18];

Method for determination of nitrites - VLM - NO_2 - №3, technical means – photometer "NOVA 60 A" ID № 08450505 [19];

Method for determination of ammonium ions - VLM - NH_4 - №1, technical means – photometer "NOVA 60 A" ID № 08450505 [17];

Method for determination of total hardness - ISO 6058 [6];

Method for determination of sulphates - VLM - SO_4 - №4, technical means – photometer "NOVA 60 A" ID № 08450505 [20];

Method for determination of calcium - ISO 6058 [6];

Method for determination of magnesium - BS 7211: 1982 [8];

Method for determination of phosphates - VLM - PO_4 - №5, technical means – photometer "NOVA 60 A" ID № 08450505 [21];

Method for determination of manganese - VLM-Mn - №7, technical means - photometer "NOVA 60 A" ID № 08450505 [23];

Method for determination of iron - VLM - Fe - № 6, technical means - photometer "NOVA 60 A" ID № 08450505 [22];

Method for determination of fluoride - VLM - F - № 8, technical means - photometer "NOVA 60 A" ID № 08450505 [24];

Method for determination of electrical conductivity - BS EN 27888, technical means - conductivity inoLab cond № 720 ID 11081137 [7].

Methods for determination of microbiological indicators

Methods for microbiological indicators in accordance with Ordinance № 9/2001 Darjaven vestnik, issue 30 and Decree № 178 / 23.07.2004 on the quality of water intended for drinking purposes [15, 16].

Method for determination of *Escherichia coli* and coliform bacteria - BS EN ISO 9308-1: 2004 [10];

Method for determination of enterococci - BS EN ISO 7899-2 [13];

Method for determination of spores of sulfite reducing anaerobes - BS EN 26461-2: 2004 [11];

Method for determination of the total number of aerobic and facultative anaerobic bacteria - BS EN ISO 6222: 2002 [14];

Method for determination of *Pseudomonas aeruginosa* - BS EN ISO 16266: 2008 [12];

Determination of coli - titre using fermentation method - the method of Ginchev;

Determination of coli - bacteria on Endo medium – a membrane method;

Determination of sulfite reducing anaerobic bacteria (*Clostridium perfringens*) – a membrane method.

Processing of the results

Data from experiments performed in triplicates are processed with the software MS Office Excel 2003 and Origin Pro 8.1, using statistical functions to determine the standard deviation and maximum error in the assessment of significance levels $\alpha < 0{,}05$.

III. Results and Discussion

A comparative physicochemical analysis of spring waters in Haskovo region focusing on the key indicators (colour according to the Rublyovska Scale, odor at 20°C, turbidity, pH, oxidation, chlorides, nitrates, nitrites, ammonium, total hardness, sulphate, calcium, magnesium, phosphates, manganese, iron, fluoride, conductivity) is conducted. The results of these studies are shown in Table 1.

Experimental data show that only spring water around the village of Yabalkovo, "Osmanova cheshma", spring water in the village of Dobrich meet all the controlled parameters of Ordinance № 9/2001 Darjaven Vestnik, issue 30 and Decree № 178/23.07.2004 on the quality of water intended for drinking purposes.

Table 1. *Physicochemical properties of the spring waters in Haskovo region*

Controlled parameter	Measuring unit	Maximum value limit	Results Haskovski min.bani 1	Results Haskovski min.bani 2	Results Gorski Izvor	Results Yabalkovo	Results Dobrich	Results Radievo
1.Colour according to the Rublyovska Scale	Degrees color	Acceptable for consumers	Acceptable	Acceptable	Acceptable	Acceptable	Acceptable	Acceptable
2.Odor at 20°C	Total	Acceptable for consumers	Acceptable	Acceptable	Acceptable	Acceptable	Acceptable	Acceptable
3.Turbidity	NTU	Acceptable for consumers	Unacceptable	Unacceptable	Acceptable	Acceptable	Acceptable	Acceptable
4.pH	pH	≥ 6,5 и ≤ 9,5	6,8	6,9	6,7	7,4	6,8	6,9
5.Oxidation	mgO_2/dm^3	5,0	0,7	0,6	1,7	0,6	1,3	0,7
6.Chlorides	mg/ dm^3	250	46	49	94	23	20	26
7.Nitrates	mg/ dm^3	50	6	10	171	6	17	84
8.Nitrites	mg/ dm^3	0,50	0,04	0,03	0,02	0,01	0,01	0,02
9.Ammoniumions	mg/ dm^3	0,50	0,13	0,12	0,18	0,04	0,05	0,08
10.Total hardness	$mgekv/ dm^3$	12	10	10	13	3	11	11
11.Sulfates	mg/ dm^3	250	723	72	173	165	97	272
12.Calcium	mg/ dm^3	150	158	164	194	30	102	144
13.Magnesium	mg/ dm^3	80	20	22	44	21	74	46
14.Phosphates	mg/ dm^3	0,5	0,1	0,1	0,5	0,1	0,4	0,0
15.Manganese	mg/ dm^3	50	1024	844	34	8	8	35
16.Iron	$\mu g/ dm^3$	200	845	533	24	16	30	20
17.Fluorides	mg/ dm^3	1,5	2,5	2,9	0,7	1,3	0,1	1,0
18.Conductivity	$\mu S/ dm^3$	2000	1966	1962	1524	841	970	1194

Table 2. *Total mesophilic aerobic and facultative anaerobic bacteria*

Studied source	Indicator, cfu/cm^3
1. Healing spring in the village of Mineralni bani	5 ± 1
2. Healing thermal spring in the village of Mineralni bani with water temperature 57°C	0
3. Spring around the village of Gorski Izvor	$1.4x10^2 – 1.6x10^2$
4. Spring around the village of Yabalkovo	7 – 9
5. Spring around the village of Dobrich	7 – 9
6. Spring around the village of Radievo	6 – 7

The microbiological indicators of the same spring waters are determined using a membrane process. Table 2 presents the experimental studies for the determination of the total number of mesophilic aerobic and facultative anaerobic bacteria in spring waters in the Haskovo region.

According to the standard requirements the tested water samples from the six springs is clean, except for the spring water around the village of Gorski izvor which contains $1x10^2 cfu/cm^3$ and belongs to the group of questionably clean waters.

The presence of coliforms and *Escherichia coli* is determined by a membrane method and the method of Ginchev. The test results (Table 3 and Table 4) show that the healing spring in the village of Mineralni bani, the thermal healing spring in the village of Mineralni bani with water temperature of 57°C and the spring around the village of Radievo meet the standard requirements for the presence of coliform bacteria. These results are confirmed by the membrane method (Table 4). All other parameters are determined by the membrane method.

Based on the conducted physicochemical and microbiological research it was found that from the six investigated springs in the Haskovo region the spring water around the village of Yabalkovo, "Osmanova cheshma" and the spring water around the village of Dobrich meet all the controlled parameters of Ordinance № 9/2001 Darjaven Vestnik, issue 30 and Decree № 178/23.07.2004 of the quality of water intended for drinking purposes when it comes to the physicochemical parameters; and the non-thermal healing spring water in the village of Mineralni bani and the spring water around the village Radievo meet the requirements for drinking water when it comes to microbiological indicators.

Table 3. *Coli - titre of healing and spring waters*

Name of the water source	Coli - titre	Inoculation volume 50cm^3	Inoculation volume 10cm^3	Inoculation volume 10cm^3	Inoculation volume 10cm^3	Inoculation volume 10cm^3	Inoculation volume 10cm^3
1. Healing spring in the village of Mineralni bani	> 100	–	–	–	–	–	–
2. Healing thermal spring in the village of Mineralni bani with water temperature 57°C	> 100	–	–	–	–	–	–
3. Spring around the village of Gorski izvor	90	+ Acid	+ Acid	+ Acid	+ Acid and gas	+ Acid and gas	–
4. Spring around the village of Yabalkovo	80	+ Acid	+ Acid	+ Acid and gas	+ Acid and gas	+ Acid and gas	–
5. Spring around the village of Dobrich	70	+ Acid	+ Acid	+ Acid and gas	+ Acid and gas	+ Acid and gas	+ Acid and gas
6. Spring around the village of Radievo	> 100	–	–	–	–	–	–

Table 4. *Microbiological indicators of spring waters in Haskovo region*

Indicators	Units	Healing spring in the village of Mineralni bani	Healing thermal spring in the village of Mineralni bani with water temperature 57°C	Spring around the village of Gorski izvor	Spring around the village of Yabalkovo	Spring around the village of Dobrich	Spring around the village of Radievo
Coli forms	cfu/cm^3	0/100	0/100	2/100	1/100	20/100	0/100
Escherichia coli	cfu/cm^3	0/100	0/100	2/100	1/100	20/100	0/100
Enterococci	cfu/cm^3	0/100	8/100	0/100	9/100	3/100	0/100
Sulfite reducing anaerobic bacteria *(Clostridium perfringens)*	cfu/cm^3	0/100	0/100	0/100	0/100	0/100	0/100

Pseudomonas aeruginosa	cfu/cm^3	0/250	0/250	0/250	0/250	0/250	0/250

References

[1] BS 8451: 1977 - determination of the color according to the Rublyovska Scale, determination of odor at 20°C.
[2] BS EN ISO 7027 - Determination of turbidity.
[3] BS 3424: 1981 - Determination of pH.
[4] BS 3413: 1981 - Determination of oxidation.
[5] BS 3414: 1980 - Determination of chlorides.
[6] BS ISO 6058 - Determination of calcium, total hardness.
[7] BS EN 27888 - Determination of electrical conductivity.
[8] BS 7211: 1982 - Determination of magnesium.
[9] BS EN ISO 7899-2 - Determination of nitrates.
[10] BS EN ISO 9308-1: 2004 - Determination of *Escherichia coli* and coliform bacteria.
[11] BS EN 26461-2: 2004 - Determination of sulfite reducing anaerobic bacteria (*Clostridium perfringens*).
[12] BS EN ISO 16266 - Determination of *Pseudomonas aeruginosa*.
[13] BS EN ISO 7899-2 - Determination of enterococci.
[14] BS EN ISO 6222: 2002 - Determination of total aerobic and facultative anaerobic bacteria.
[15] Decree № 178/23.07.2004 on the quality of water intended for drinking purposes.
[16] Ordinance № 9/2001 Darjaven vestnik, issue 30.
[17] VLM - NH_4 - № 1 - Determination of ammonium ions.
[18] VLM - NO_3 - № 2 - Determination of nitrates.
[19] VLM - NO_2 - № 3 - Determination of nitrites.
[20] VLM - SO_4 - № 4 - Determination of sulfates.
[21] VLM - PO_4 - № 5 - Determination of phosphates.
[22] VLM - Fe - № 6 - Determination of iron.
[23] VLM - Mn - № 7 - Determination of manganese.
[24] VLM - F - № 8 - Determination of fluorides.

ANTIMICROBIAL ACTIVITY OF A PROBIOTIC STRAIN *LACTOBACILLUS ACIDOPHILUS* A2 OF HUMAN ORIGIN AGAINST PATHOGENS

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Abstract. *The antimicrobial activity of the strain Lactobacillus acidophilus A2 against the pathogens E.coli ATCC 25922, E.coli ATCC 8739, Salmonella abony NTCC 6017, Salmonella sp., Staphylococcus aureus ATCC 25293 and Proteus vulgaris J is determined by joint cultivation at 37 ± 1°C. It is shown that the Lactobacillus strain inhibits the growth of the pathogens - for 36 - 72 hours the numbers of viable cells of the pathogens are reduced. It has been shown that changes in the proportions in the mixed population are a result of the formed lactic acid which acidifies the environment and changes the conditions for the growth of the pathogens.*

Key words: *Lactobacillus*, Pathogen, Joint cultivation, Probiotic, *E.coli*, *Salmonella*, *Staphylococcus*, *Proteus*

I. Introduction

Probiotics are living microorganisms that have beneficial effects on the host, when administered in appropriate amounts [2, 4]. Their beneficial effects in gastro-intestinal infections, reduction of serum cholesterol, inhibition of infection caused by *Helicobacter pylori*, Crohn's disease, restoration of the microflora in the stomach and intestine after antibiotic treatment, protecting the immune system, their anti-cancer properties, antimutagenic effect, antidiarrheal properties and others have been proven [1, 3, 7, 9].

Lactobacilli and bifidobacteria are natural components of the gastrointestinal microflora of a healthy person. They are included in the composition of probiotics and probiotic foods, given their proven health effect on the body [6]. They are the main microorganisms that maintain the balance of the gastrointestinal microflora [8].

Not all strains of lactobacilli and bifidobacteria may be used as components of probiotics or probiotic foods, but only those which meet certain requirements: to be of human origin; to be non-pathogenic, to be resistant to gastric juice, bile salts, and to allow implementation of technological processes during which they accumulate high concentrations of viable cells; to have the potential to adhere to the gastrointestinal epithelium; to produce antimicrobial substances; to be resistant to the existing antibiotics in medical practice; to allow industrial cultivation, encapsulation, freeze-drying and to retain their activity during storage [5]. This requires the mandatory selection of bifidobacteria and lactobacilli strains with probiotic properties.

One of the requirements for probiotic strains is to possess antimicrobial activity against conditionally pathogenic, carcinogenic and pathogenic microorganisms, which is associated with inactivation of their enzyme systems, overcoming their adhesion, inhibiting their growth and ejection of the pathogens from the biological niche resulting in normal gastrointestinal microflora.

The purpose of the present study is to determine the antimicrobial activity of *Lactobacillus acidophilus* A2 of human origin against the pathogens *E.coli* ATCC 25922, *E.coli* ATCC 8739, *Salmonella abony* NTCC 6017, *Salmonella* sp., *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris* J by joint cultivation of *Lactobacillus acidophilus* A2 and each of the pathogens.

II. Materials and Methods

2.1. Media

Sterile skimmed milk with titratable acidity 16-18ºT. Composition (g/dm^3): skimmed milk powder (Scharlau). Sterilization - 15 minutes at 118ºC.

Saline solution. Composition (g/dm^3): NaCl - 5. Sterilization - 20 minutes at 121ºC.

LAPTg10-broth. Composition (g/dm^3): peptone - 15, yeast extract - 10; tryptone - 10, glucose - 10. pH is adjusted to 6.6 - 6.8 and Tween 80 - 1cm^3/dm^3 is added. Sterilization - 20 minutes at 121ºC.

LAPTg10-agar. Composition (g/dm^3): LAPTg10-broth + 2% agar. Sterilization - 20 minutes at 121ºC.

LBG-agar. Composition (g/dm^3): Tryptone – 10, yeast extract - 5, NaCl – 10, glucose – 10, agar – 2%. pH is adjusted to 7.5. Sterilization - 20 minutes at 121°C.

2.2. Determination of the antimicrobial activity of Lactobacillus acidophilus A2 against pathogenic microorganisms

To determine the antimicrobial activity of *Lactobacillus acidophilus* A2 against pathogens a 24 hour culture of *Lactobacillus acidophilus* A2 developed in skimmed milk is used. In the mixtures are mixed 0.5 ml of the suspension of the *Lactobacillus* strain, 0.5 ml of the suspension of the pathogen and 9 ml of culture medium (skimmed milk), and in the controls of the *Lactobacillus* strain or the respective pathogen 9.5 ml culture medium (skimmed milk) is mixed with 0.5 ml of the suspension of the *Lactobacillus* strain or the pathogen, respectively. Joint cultivation of *Lactobacillus acidophilus* A2 and each of the pathogens *E.coli* ATCC 25922, *E.coli* ATCC 8739, *Salmonella abony* NTCC 6017, *Salmonella* sp., *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris* J under static conditions in a thermostat at 37±1°C for 72 hours, taking samples at 0, 12, 24, 36, 48, 60 and 72 hours is conducted and the change in the titratable acidity and the concentration of viable cells of the pathogen and of the *Lactobacillus* strain is monitored.

III. Results and Discussion

The antimicrobial activity of *Lactobacillus acidophilus* A2 against the pathogens *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella* sp., *Salmonella abony* NTCC 6017, *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris* J is examined by joint and separate cultivation at 37±1ºC in skimmed milk.

During separate cultivation *Lactobacillus acidophilus* A2 and each of the pathogens, included in the study, accumulate high concentrations of viable cells for 72 hours of incubation (10^{13} - 10^{15}cfu/cm^3). The control of *Lactobacillus acidophilus* A2 reach higher titratable acidity (309.69°T) in comparison with the titratable acidity of the controls of the pathogens that increases slightly during the separate cultivation, reaching values between 29.76°T and 40.92°T.

In joint cultivation of *Lactobacillus acidophilus* A2 and *E.coli* ATCC 25922 or *E.coli* ATCC 8739 at 37±1°C under static conditions in skimmed milk, an increase in the concentration of viable cells of *Lactobacillus acidophilus* A2, *E.coli* ATCC 25922 and *E.coli* ATCC 8739 in the first 12 hours is observed.

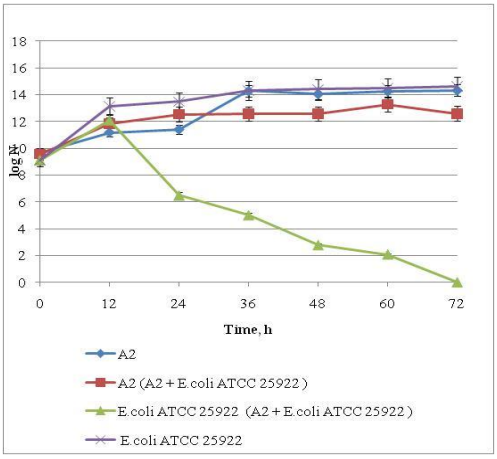

Fig. 1. Survival of *Lb.acidophilus* A2 and *E.coli* ATCC 25922 during separate cultivation and cultivation in a mixed population at 37±1°C.

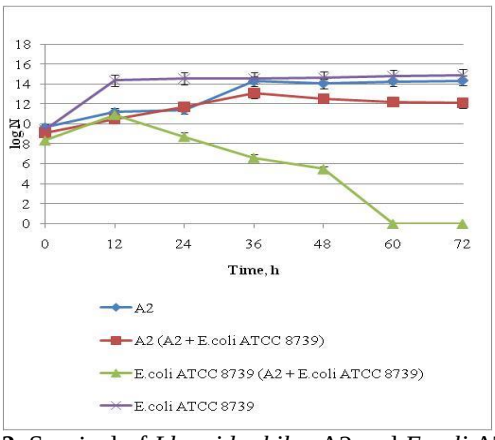

Fig. 2. Survival of *Lb.acidophilus* A2 and *E.coli* ATCC 8739 during separate cultivation and cultivation in a mixed population at 37±1°C.

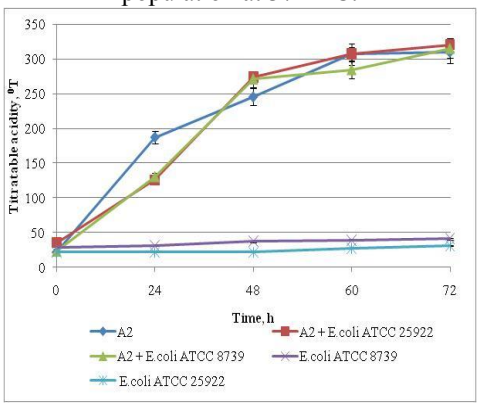

Fig. 3. Change in the titratable acidity of the medium during joint and separate cultivation of *Lb.acidophilus* A2 and *E.coli* ATCC 25922 or *E.coli* ATCC 8739 at 37±1°C.

After that the concentration of viable lactobacilli cells continues to increase, while that of the pathogen is reduced and by the 60th hour for *E.coli* ATCC 8739 (Fig. 2) and by the 72nd hour for *E.coli* ATCC 25922 (Fig. 1) no living cells of the pathogen are observed.

In the cultivation of *Lactobacillus acidophilus* A2 and *Salmonella* sp. (clinical isolate) at 37±1°C, an increase in the concentration of viable cells of *Lactobacillus acidophilus* A2 and *Salmonella* sp. during the first 24 hours is observed, after which the concentration of viable lactobacilli cells continues to increase, while that of the pathogen is reduced and by the 72nd hour no living cells of *Salmonella* sp. are defined (Fig. 4).

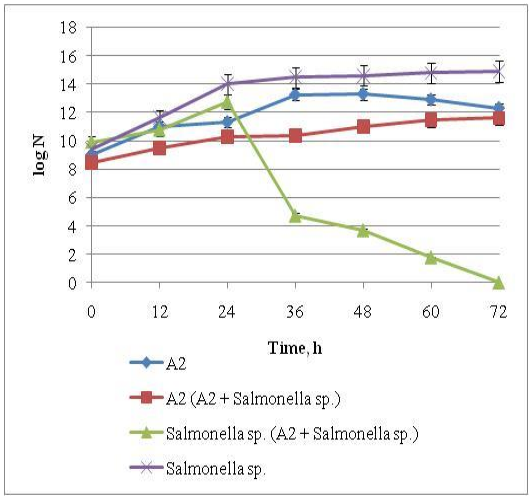

Fig. 4. Survival of *Lactobacillus acidophilus* A2 and *Salmonella* sp. during separate cultivation and in a mixed population at 37±1°C.

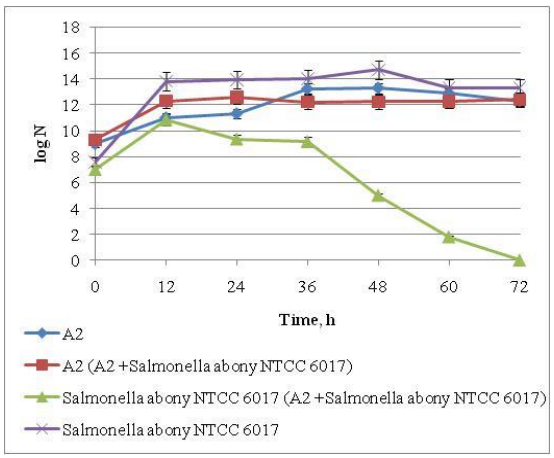

Fig. 5. Survival of *Lactobacillus acidophilus* A2 and *Salmonella abony* NTCC 6017 during separate cultivation and cultivation in a mixed population at 37±1°C.

During the cultivation of *Salmonella abony* NTCC 6017 and *Lactobacillus acidophilus* A2 in a mixed population an increase in the concentration of lactobacilli cells and of the pathogen cells in the first 12 hours is established. But between the 12th and the 72nd hour the concentration of lactic acid bacteria remains almost unchanged, while that of *Salmonella abony* NTCC 6017 decreases and at the 72nd hour no living cells of the pathogen are detected (Fig. 5).

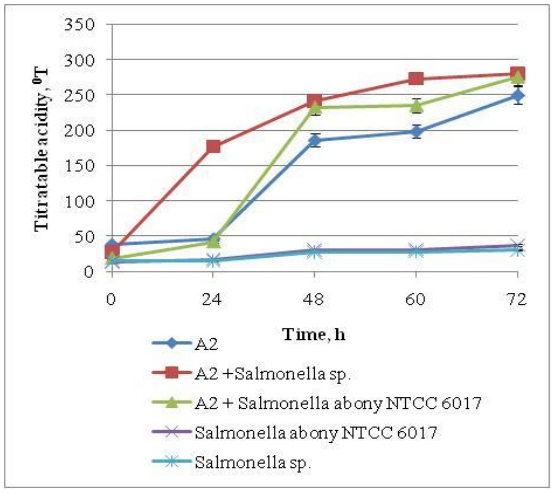

Fig. 6. Change of the titratable acidity of the medium in joint and separate cultivation of *Lactobacillus acidophilus* A2 and *Salmonella abony* NTCC 6017 or *Salmonella* sp. at 37±1°C.

During separate cultivation *Lactobacillus acidophilus* A2 and *Staphylococcus aureus* ATCC 25293 accumulate high concentrations of active cells. In the joint cultivation of *Lactobacillus acidophilus* A2 and *Staphylococcus aureus* ATCC 25293 a decrease in the concentration of the pathogen, starting after the 24th hour is observed. In contrast to *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella* sp. and *Salmonella abony* NTCC 6017 this pathogen is not reduced completely in the presence of *Lactobacillus acidophilus* A2. At the end of the process $3.2x10^2$cfu/cm^3 viable cells of the pathogen are detected (Fig. 7). Despite the high titratable acidity (Fig. 8), because of the accumulation of organic acids in the medium, *Lactobacillus acidophilus* A2 does not suppress the pathogen completely.

In joint cultivation of *Lactobacillus acidophilus* A2 and *Proteus vulgaris* J at 37±1°C under static conditions, there is an increase in the concentration of viable cells of *Lactobacillus acidophilus* A2 and *Proteus vulgaris* J in the first 12 hours, after which the concentration of viable lactobacilli cells continues to

increase, while that of the pathogen is reduced and at the 36th hour no living cells of *Proteus vulgaris* J are detected (Fig. 9).

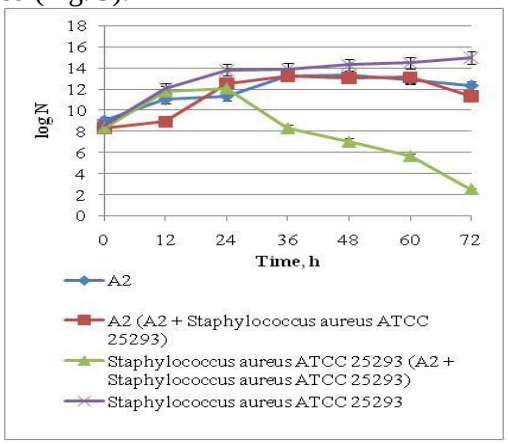

Fig. 7. Survival of *Lactobacillus acidophilus* A2 and *Staphylococcus aureus* ATCC 25293 during separate cultivation and cultivation in a mixed population at 37±1°C.

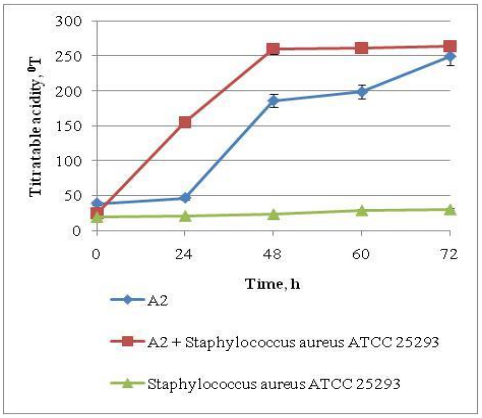

Fig. 8. Change in the titratable acidity of the medium during separate cultivation and cultivation in a mixed population at 37±1°C of *Lactobacillus acidophilus* A2 and *Staphylococcus aureus* ATCC 25293.

The complete reduction of viable cells of the pathogens *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella* sp, *Salmonella abony* NTCC 6017 and *Proteus vulgaris* J is largely a result of the increased titratable acidity due to the accumulation of lactic acid and other organic acids in the medium (Fig. 3, Fig. 6, Fig. 10).

IV. Conclusion

Lactobacillus acidophilus A2 demonstrates antimicrobial activity against *E.coli* ATCC 25922, *E.coli* ATCC 8739, *Salmonella abony* NTSS 6017, *Salmonella* sp., *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris* J. In joint cultivation *Lactobacillus acidophilus* A2 retains high concentrations of viable cells, while the number of the cells of the pathogen reduces and the degree of reduction is strain specific and is partly due to the change in the acidity of the medium as a result of the acid production by *Lactobacillus acidophilus* A2.

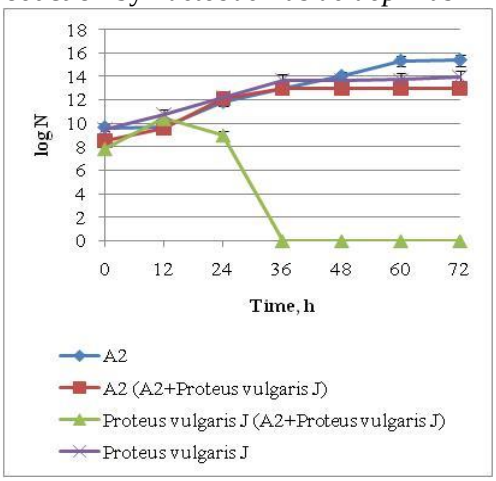

Fig. 9. Survival of *Lactobacillus acidophilus* A2 and *Proteus vulgaris* J during separate cultivation and cultivation in a mixed population at 37±1°C.

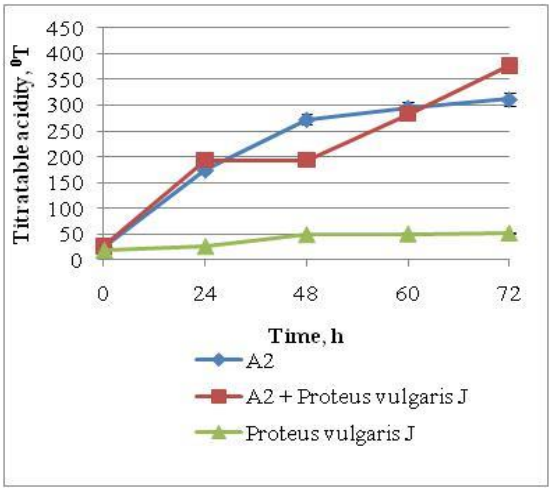

Fig. 10. Change in the titratable acidity of the medium during separate cultivation and cultivation in a mixed population at 37±1°C of *Lactobacillus acidophilus* A2 and *Proteus vulgaris* J at 37±1°C.

The antimicrobial activity against pathogens makes *Lactobacillus acidophilus* A2 a potential probiotic

strain and after further evaluations they may be included in the formulation of probiotic preparations for prophylaxis and treatment.

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INHIBITORY ACTIVITY OF THE PROBIOTIC STRAIN *BIFIDOBACTERIUM BIFIDUM* BIF. 4 OF HUMAN ORIGIN AGAINST PATHOGENS

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Abstract. *The antimicrobial activity of the strain Bifidobacterium bifidum Bif. 4 against the pathogens E.coli ATCC 25922, E.coli ATCC 8739, Salmonella abony NTCC 6017, Salmonella sp., Staphylococcus aureus ATCC 25293 and Proteus vulgaris J is determined by joint cultivation at 37±1°C. It is shown that Bifidobacterium bifidum Bif. 4 inhibits the growth of the pathogens - for 60 - 72 hours the numbers of viable cells of the pathogens are reduced. It has been shown that the changes in the proportions in the mixed population are due to the accumulation of lactic and acetic acids which acidify the environment and change the conditions for the growth of the pathogens, leading to reduction of the number of viable cells of the pathogens.*

Key words: Pathogen, Joint cultivation, Probiotic, *Bifidobacterium*, *E.coli*, *Salmonella*, *Staphylococcus*, *Proteus*

I. Introduction

Maintaining the balance of the microflora in the stomach and intestines is necessary for good health. Restoring the balance of the intestinal microflora is achieved by the intake of food and concentrates containing beneficial bacteria - lactobacilli and bifidobacteria, known as functional foods and probiotics, respectively. FAO defines probiotics as live microorganisms that have beneficial effect on the host when administered in adequate amounts [2, 3].

The main components of probiotics are lactic acid bacteria (*Lactobacillus*, *Enterococcus*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*) and bifidobacteria, which are applied in the manufacture of probiotic foods as well [4, 7, 8, 9, 12], the largest proportion being the lactobacilli. Not all lactobacilli can be included in the composition of probiotics and probiotic foods, but only those who possess certain properties [6, 7, 8]: to be a part of the natural microflora in humans and animals; to have the ability to adhere to epithelial cells or cell lines; to be able to survive under the conditions in the stomach and the intestines (acidic pH in the stomach and bile) [5, 11]; to be able to reproduce in the intestinal tract; to be able to suppress and expel pathogenic and toxicogenic microorganisms from the biological niche; to allow industrial cultivation; to have antimicrobial activity against conditionally pathogenic, carcinogenic and pathogenic microorganisms; to produce antimicrobial substances; to modulate the immune response and to be safe for clinical and food applications [6, 7, 8].

The studies of Saxelin et al., (1996 a, b), Donohue & Salminen, (1996), Salminen et al., (1998) [1, 6, 7, 8, 9, 10] demonstrate the safety of the lactic acid bacteria and bifidobacteria and strains belonging to the genera *Lactobacillus*, *Lactococcus* and *Bifidobacterium* most often are with GRAS status.

The purpose of the present paper is to determine the antimicrobial activity of a probiotic strain of human origin *Bifidobacterium bifidum* Bif.4 against the following pathogens: *E.coli* ATCC 25922, *E.coli* ATCC 8739, *Salmonella abony* NTCC 6017, *Salmonella* sp., *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris* J.

II. Materials and Methods

2.1. Media

Sterile skimmed milk with titratable acidity 16-18ºT. Composition (g/dm^3): skimmed milk powder (Scharlau). Sterilization - 15 minutes at 118ºC.

Saline solution. Composition (g/dm^3): NaCl - 5. Sterilization - 20 minutes at 121ºC.

LBG-agar. Composition (g/dm^3): Tryptone – 10, yeast extract - 5, NaCl – 10, glucose – 10, agar – 2%. pH is adjusted to 7.5. Sterilization - 20 minutes at 121°C.

Solid medium for bifidobacteria. Ingredients (g/dm^3): peptone - 10, yeast extract - 10 lactose - 10, $MnSO_4$ – 1, casein hydrolyzate - 8, NaCl - 3.2,

CH_3COONa – 1, agar – 2%. pH is adjusted to 6.6 - 6.8. Sterilization - 20 minutes at 121°C.

2.2. Determination of the antimicrobial activity against pathogenic microorganisms

To determine the antimicrobial activity of *Bifidobacterium bifidum* Bif.4 against pathogens a 24 hour culture of the tested strain developed in skimmed milk is used. In the mixtures are mixed 0.5 ml of the suspension of *Bifidobacterium bifidum* Bif.4, 0.5 ml of the suspension of the pathogen and 9 ml of culture medium (skimmed milk), and in the controls of the *Bifidobacterium* strain or the pathogen 9.5 ml culture medium (skimmed milk) is mixed with 0.5 ml of the suspension of the *Bifidobacterium* strain or the pathogen, respectively. Joint cultivation of *Bifidobacterium bifidum* Bif.4 and each of pathogens *E.coli* ATCC 25922, *E.coli* ATCC 8739, *Salmonella abony* NTCC 6017, *Salmonella* sp., *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris* J under static conditions in a thermostat at 37±1°C for 72 hours, taking samples at 0, 12, 24, 36, 48, 60 and 72 hours is conducted and the changes in the titratable acidity and the concentration of viable cells of the pathogen and of the *Lactobacillus* or the *Bifidobacterium* strain are monitored.

2.3. Processing of the results

Data from triplicate experiments is processed using the software MS Office Excel 2003 and Origin Pro 8.1, using statistical functions to determine the standard deviation and maximum error of assessment in the significance level of $\alpha < 0{,}05$.

III. Results and Discussion

The antimicrobial activity of *Bifidobacterium bifidum* Bif. 4 against the pathogens *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella* sp. (clinical isolate), *Salmonella abony* NTCC 6017, *Staphylococcus aureus* ATCC 25293, *Proteus vulgaris* J during joint and separate cultivation at 37±1ºC in skimmed milk is determined.

During separate cultivation in skimmed milk *Bifidobacterium bifidum* Bif. 4 and all the pathogens included in the study accumulate high concentrations of viable cells. But there are differences in the mixed populations. In joint cultivation of *Bifidobacterium bifidum* Bif. 4 and *E.coli* ATCC 25922 there are changes in the microbial population. The presence of the pathogenic microorganism has no influence on the growth of the bifidobacteria and its concentration of viable cells exceeds 10^{13}cfu/cm^3 at the 24th hour and remains almost constant throughout the cultivation period of 72 hours while the concentration of active cells of the pathogen starts decreasing after the 12th hour and at the 60th hour no living cells of *E.coli* ATCC 25922 are established (Fig. 1).

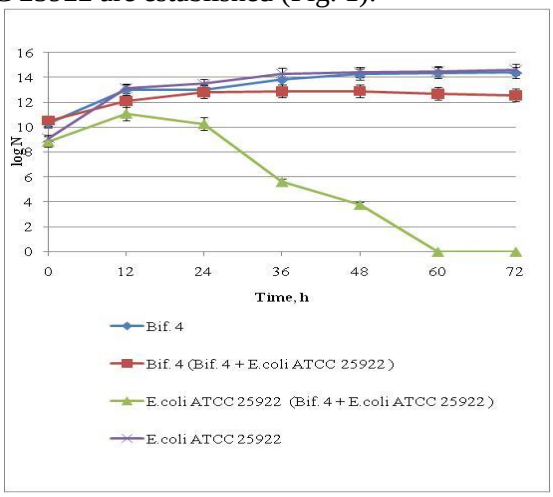

Fig. 1. Survival of cells of *Bifidobacterium bifidum* Bif. 4 and *Escherichia coli* ATCC 25922 during separate cultivation and cultivation in a mixed population at 37± 1º C.

Similar results are observed in the joint cultivation of *Bifidobacterium bifidum* Bif. 4 and the other representative of the coli bacteria *Escherichia coli* ATCC 8739 (Fig. 2). The reduction of viable cells of the pathogen starts after the 12th hour and at the 60th hour from the beginning of the process living cells of the pathogen are not detected. The value of the titratable acidity at the 60th hour is 189,26ºT (Fig. 13).

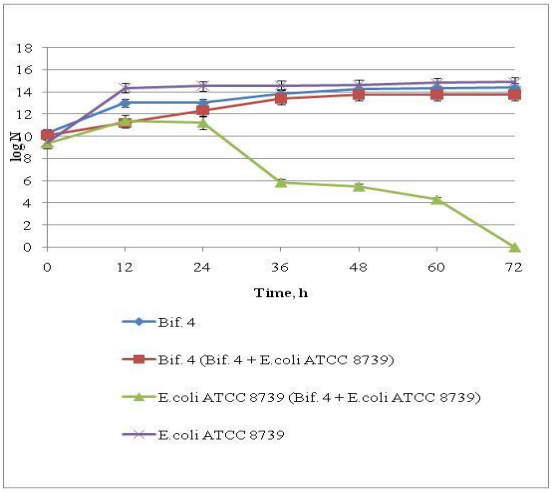

Fig. 2. Survival of cells of *Bifidobacterium bifidum* Bif. 4 and *Escherichia coli* ATCC 8739 during separate cultivation and cultivation in a mixed population at 37± 1º C.

The presence of bifidobacteria in the medium has a substantial influence on the growth of the pathogen

and after the 12th hour the viable pathogen cell concentration is quickly reduced up to the 60th hour when no living cells are determined. This is due to the production of lactic and acetic acids *Bifidobacterium bifidum* Bif. 4 which decrease the pH of the medium and suppress the growth of the pathogens (Fig. 13).

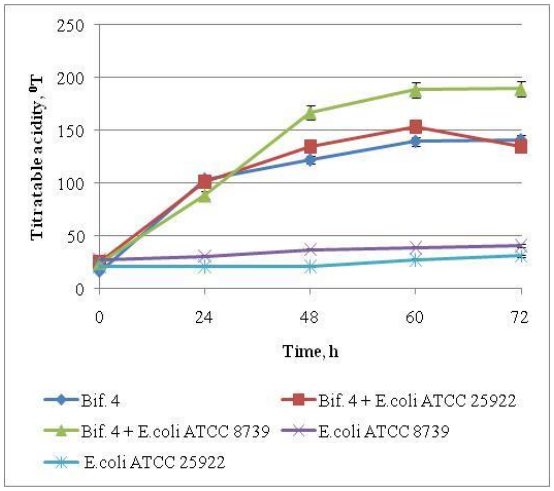

Fig. 3. Change of the titratable acidity of the medium in joint cultivation and cultivation in a mixed population of *Bifidobacterium bifidum* Bif. 4 and the pathogenic microorganisms - *E. coli* ATCC 25922 and *E. coli* ATCC 8739 at 37±1°C.

The inhibitory activity of *Bifidobacterium bifidum* Bif. 4 against *Salmonella* bacteria is examined. During joint cultivation *Salmonella abony* NTCC 6017 and the bifidobacteria an increase in the concentration of viable cells in the first 12 hours is observed.

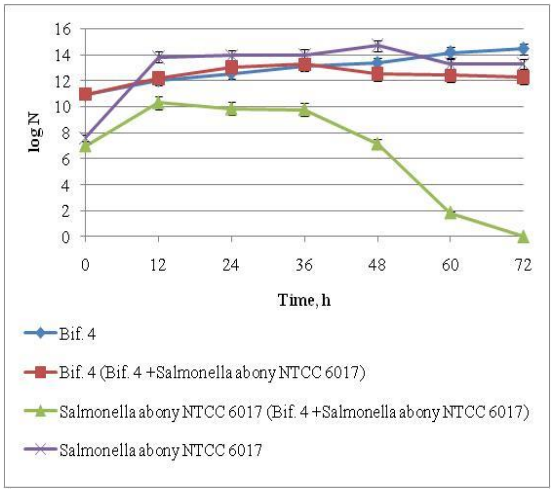

Fig. 4. Survival of cells and *Bifidobacterium bifidum* Bif. 4 and *Salmonella abony* NTCC 6017 during separate cultivation and cultivation in a mixed population at 37± 1° C.

After that the concentration of viable cells of the bifidobacteria is maintained, whereas that of the pathogen is reduced at a slower rate at first, and then more quickly and at the 72nd hour there are no living pathogen cells (Fig. 4).

In joint cultivation of *Bifidobacterium bifidum* Bif. 4 and the other representative of *Salmonella Salmonella* sp. (clinical isolate) an increase in the number of viable cells in the first 12 hours for both strains is observed and after that an increase in the number of bifidobacteria living cells is established. The cells of the pathogen are reduced, reaching 0 at the 72nd hour (Fig. 5).

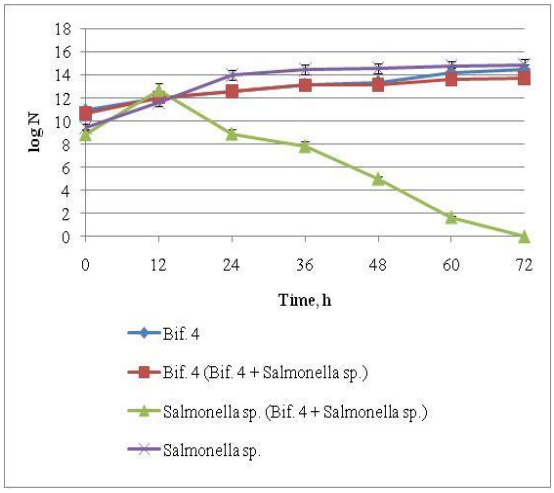

Fig. 5. Survival of cells and *Bifidobacterium bifidum* Bif. 4 and *Salmonella* sp. during separate cultivation and cultivation in a mixed population at 37± 1° C.

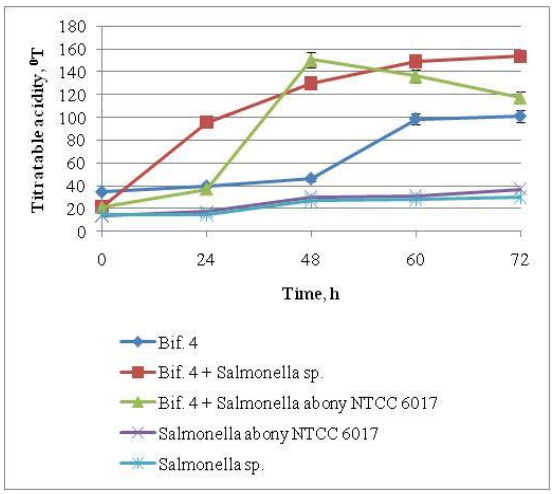

Fig. 6. Change of the titratable acidity of the medium in joint cultivation and cultivation in a mixed population of *Bifidobacterium bifidum* Bif. 4 and the pathogenic microorganisms - *Salmonella abony* NTCC 6017 and *Salmonella* at 37±1°C.

The antimicrobial activity of *Bifidobacterium bifidum* Bif. 4 against *Staphylococcus aureus* ATCC 25293 is studied. It is found that the content of viable cells of *Bifidobacterium bifidum* Bif. 4 and the pathogen increases in the first 36 hours, after which the number of viable cells of *Bifidobacterium bifidum* Bif. 4 is maintained. The cells of the pathogen decrease rapidly, reaching 0 at the 72nd hour (Fig. 7).

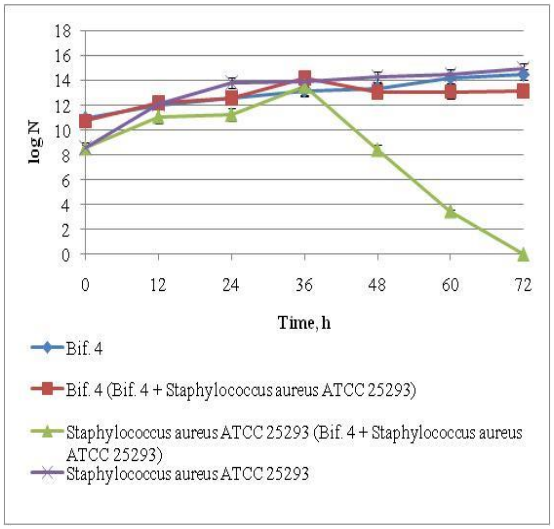

Fig. 7. Survival of cells and *Bifidobacterium bifidum* Bif. 4 and *Staphylococcus aureus* ATCC 25293 during separate cultivation and cultivation in a mixed population at 37± 1° C.

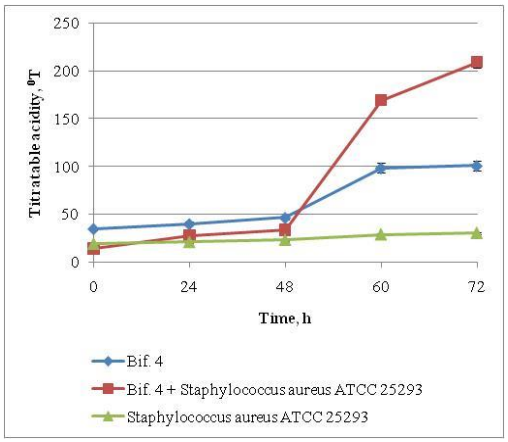

Fig. 8. Change of the titratable acidity of the medium in joint cultivation and cultivation in a mixed population of *Bifidobacterium bifidum* Bif. 4 and *Staphylococcus aureus* ATCC 25293 at 37±1°C.

During the joint development of *Bifidobacterium bifidum* Bif. 4 and *Proteus vulgaris* J the concentration of viable cells of the bifidobacteria and the pathogen increase in the first 12 hours. After that *Bifidobacterium bifidum* Bif. 4's cell concentration continues to grow, while that of the pathogen reduces. At the 60th hour 1.10^2cfu/cm^3 live cells of pathogenic bacteria are defined (Fig. 9).

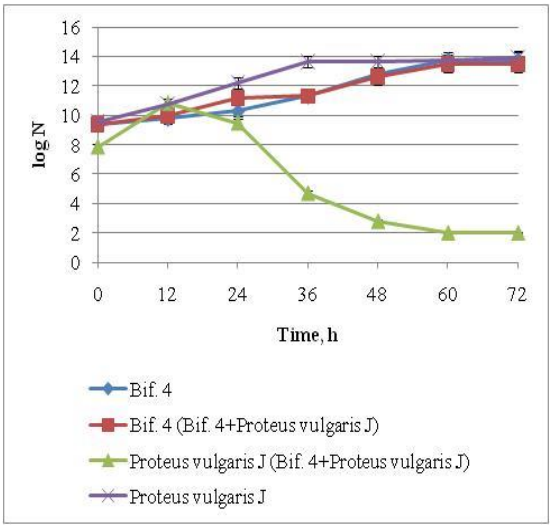

Fig. 9. Survival of *Bifidobacterium bifidum* Bif. 4 and *Proteus vulgaris* J during separate cultivation and cultivation in a mixed population at 37± 1° C.

The acidity of the control of the pathogenic microorganisms is lower than that of the control of *Bifidobacterium bifidum* Bif. 4 and the acidity of the mixture. Inhibition by *Bifidobacterium bifidum* Bif. 4 to a significant degree is due to the decrease in the pH of the medium as a result of the produced lactic and acetic acid (Fig. 3, Fig. 6, Fig. 8, Fig. 10).

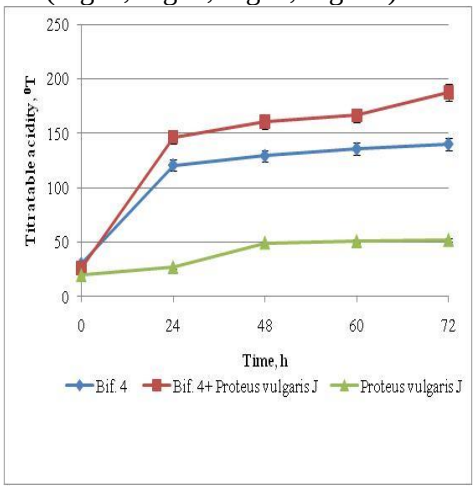

Fig. 10. Change of the titratable acidity of the medium in joint cultivation and cultivation in a mixed population of *Bifidobacterium bifidum* Bif. 4 and *Proteus vulgaris* J at 37±1°C.

IV. Conclusion

The strain *Bifidobacterium bifidum* Bif.4 inhibits the growth of pathogens *E.coli* ATCC 25922, *E.coli* ATCC 8739, *Salmonella abony* NTCC 6017, *Salmonella* sp., *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris* J. During joint cultivation *Bifidobacterium bifidum* Bif.4 retains a high concentration of viable cells. The number of cells of the pathogens are reduced and the degree of reduction is strain specific. The antimicrobial activity against pathogens makes the strain *Bifidobacterium bifidum* Bif.4 a potentially probiotic one.

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REQUIREMENTS FOR HYGIENIC DESIGN OF TECHNOLOGICAL EQUIPMENT IN THE FOOD INDUSTRY

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Abstract. *The paper presents the requirements for hygienic design for production equipment used in the food industry. The presented requirements can serve as basic criteria for equipment manufacturers and the organizations that purchase technological equipment.*

Keywords: hygienic design, technological equipment, biological, chemical and physical hazards

I. Introduction

Maintaining a high level of hygiene of the equipment is essential for the safety of food products during their production. There are two methods for maintaining the hygiene of a food process. One is required a complete or partial disassembly and manual cleaning of every component – so called Cleaning out of place (COP). During 1950's has been developed a method to clean the equipment without dismantling – Cleaning in Place (CIP). This method has benefit of better repeatability, reduced downtime and reduced recontamination risk. COP is still used today, but the CIP method has prevailed due on-going technical breakthroughs and development /3/. To be able to apply CIP systems, the process equipment must be designed according to design requirements for hygiene. The latter helps maintain utmost hygiene necessary for the production of safe food and beverages. If processed food is not equipment with hygienic design will be difficult to clean different types of contaminants (biological, chemical, physical, allergens and genetically modified organisms (GMOs)), which could affect product quality and safety.

Microbiological contamination of equipment can occur during processing and packaging of the product or be caused by microorganisms multiply in food residues.

Chemical contamination of the equipment may result from residual disinfectants and lubricants.

Physical contamination and contamination with allergens and GMOs may occur as a result of the so-called crossing streams or are driven by the inability to effectively clean the equipment due to failures to hygienic design.

Within the European Union was established European Group on hygienic design (EHEDG) fouded in 1989 The aim of EHEDG is to promote hygiene during the processing and packing of food product /5/. EHEDG develops guidelines to regulate the requirements for hygienic equipment design.

II. Hygienic design requirements

They are the number of hygienic design requirements to the equipment regarding its planning and production from the one hand and the selection of equipment from the business operators in food industry from the other hand.

1. Requirements for welding of stainless steel for the manufacture of containers with hygienic design

- The pipelines should have a maximum internal roughness Ra=0,8 μm;
- Tungsten-inert gas for welding should be used;
- Automated machines should be used to achieve consistently high quality results;
- The weld must be filled accurately the gap between the pipe and fitting. There should be no excess or deficiency. Do not penetrate deeper and there are no defects in the weld surface (cracks, etc.);
- The inner surface should also be protected with gas – argon should be used for this purpose;
- The ends of the pipes should be clean at the point of melting. The ends of the pipes also should be cleaned with a stainless steel brush and solvent to remove dirt and grease;
- The ends of the pipe should be properly trimmed using mechanical tools (not manually). Should no cutting burrs and distortion. If is necessary preparations before welding (wall thickness greater than 3 mm), they must be cut by hand;

- The diameter of the pipe should be the same or smaller diameter can be extended with a special tool to prevent the creation of a bad foot and welding;
- Rough places should be limited to less than 20% of the wall thickness;
- The preliminary experiments with test pieces to establish the optimal conditions for the wall thickness of the pipe it is required.

2. Hygienic design requirements for indoors equipment

- Avoiding of joints it is strongly recommended;
- Demountable connections must be sealed with a suitable sealing;
- Metal-metal connections are not suitable for hygienic design and should not be used;
- Dynamic gaskets seal well and are easy to clean, but single dynamic seals do not prevent entry of microorganisms, i. e. properly designed equipment will be hygienic, but not aseptic;
- Diaphragm or double dynamic seals for sealing of aseptic equipment should be used;
- Piping must have a slope of 3^o;
- Avoid using a T-fitting to prevent dead zones;
- The surface in contact with the product must have a roughness Ra≤0,8 μm. In some cases, a rough surface is also acceptable (producing beverages);
- All sharp internal corners and edges should be avoided in order to facilitate cleaning;
- Screws on the product contact side should be avoided. If their use is unavoidable, should be used seals in accordance with ISO 255:1983;
- Should not be used insulation with chlorine releasing agents;
- Should be performed periodic replacement o-rings systems.

3. Hygiene Requirements for various joints and elements

The characteristic parameters for the design of solid piping welds are as follows:

- Easy to clean in place (CIP);
- Sterilization in place;
- Impervious to microorganisms;
- Easy to install;
- Reliable, i. e. resistant to technological conditions between - 10°C at 2,5 MPa and 120 °C to 120 MPa, resistant to steam sterilization at 140 °C and a pressure of more than 0,3 MPa for 20 min. Compatible and resistant to the product and the cleaning and sanitation, bacteria resistant in all conditions mentioned above.

Static seals must meet the following specifications:

- Seal hardness 70° Shore A;
- Surface roughness of the metal surface – Ra≥0,8 μm;
- Contact pressure should be 1,5 N/mm^2 minimum and 2,5 N/mm^2 maximum;
- Surface porous contacted with the product: for metal parts – no porous are allowable; for seals – no pores larger than 1 μm;
- Slip during tightening should be avoided. Should be used materials with low level of wearing;
- Seal hollows on the product contact surface ≤ 0,2 mm;
- Projections of the seal at the side contacting with the product ≤ 0,2 mm;
- Avoid pressure on the seal, which would lead to crush to 20-25%;
- For incompressible sealants that can be deformed should be created a space of possibility to adjust deformed seal.

4. Hygienic design of valves

- Roughness of the product contact surface should be below 0,8 μm to avoid holes and crevices, sharp edges, dead zones;
- Seals in valves should be as less;
- Springs in contact with the product should be avoided;
- For aseptic applications the moving parts of the valves should be separated from the product by a diaphragm or membrane.

5. Hygienic design of equipment for open proceedings

5.1 The equipment coming into contact with food

- Materials should be non-toxic, mechanically stable, inert and resistant to the product, as well as all the cleaning and sanitation materials in the whole range of pressure and temperature used in production;
- Permanent equipment connections should preferably be welded;
- Round edge with radius more than 3 mm and properly welded seams adjacent are recommended for hygienic design;
- Detachable, fixed with screws or bolts should be used only if disassembly is inevitable;
- At the product contact surface there should be no bolts;
- If the connection has to be made with screws or bolts, the formation of dead zones and gaps should be avoided;

- Metal to metal contact should be avoided by using special seals;
- The food storage equipment (utensils, containers, tanks) should has drainage valves able to swell its own;
- The sharp edges and vertical sides should be avoided for good drainage.

5.1.1 Upper edges

In food storage equipment upper edges should not be curved because of the product can be kept.

5.1.2 Covers/ lids

- Must be fully removable concerning cleaning;
- Not removable lids should have a slope concerning drain.

5.1.3 Ancillary equipment

- Mixers and blenders should be placed over the area of product. The shaft should be penetrated the product instead of making a hole in the wall of the equipment;
- If the engine must be placed over the product, it should be placed away from the equipment;
- If the engine is over the product area the possibility of contamination with engine oil should be avoided using dripping traps in combination with a ring around the shaft.

5.1.4 Axle ends and connectors

Gaps coming in contact with product and created by the contact between the metal parts and dead zones in the grooves should be avoided.

5.1.5 Bearings

Bearings in the area of product should be avoided. If it can not be removed, they must be placed so as to allow free cleaning.

5.1.6 Conveyors

- Should be with covering material to avoid contact with the product and prevent penetration of liquids inside the conveyor system;
- The ends of the belt should be supplied with removable and easy to clean covers;
- Horizontal surfaces should be constructed at an angle 5º contributing drainage of liquids;
- Elements/ brackets should allow drainage of liquids.

5.1.7 Membranes, screens, grids and perforated sheets

Should be avoided the area of the product.

5.1.8 Equipment not in contact with food

- Materials for equipment which is not in contact with food should be easy to clean and resistant to the product, cleaning detergents and disinfectants;
- Miscellaneous metals should not be placed in contact with each other due to the risk of contact corrosion;
- If the components are covered (engines, gearboxes, etc.) the coverage should not be toxic and should be resistant to cracking and peeling;
- The insulation should be impervious to moisture regarding to prevent the development of microorganisms.

5.2 Lining and frames

- Should be smooth, continuous without cracks to ensure easy cleaning;
- Edges, protrusions and indentations should be avoided because they can keep pollutants;
- If possible horizontal edges and protrusions should be inclined. Minimum angle of 30 ° is needed to avoid the accumulation of dust and to allow drainage;
- Lining should allow distance between the lowest part and the floor over 30 cm;
- Unlined structures should be designed so as not to form cavities or should be rounded.

5.3 Installation

- Where auxiliary pieces of equipment attached to the floor or walls need to leave a little space for cleaning and inspection otherwise the equipment should be properly sealed;
- Walkways and stairs over the product should be avoided due to the potential contamination of clothing or footwear of staff;
- Corridors should have raised edges;
- The floor should has not a sliding surface;
- Paths should be equipped with handrails.

6. Hygienic design of sterilization systems

Sterilization systems should be corrosion resistant under normal conditions of use taking into account the properties of the product temperature sterilization and cleaning solution for cleaning temperature.

6.1 Components of the system

All materials should be eligible for food contact.

6.2 Steam injectors and delivery of steam

- Is it appropriate configuration selected (design of the injector, steam pressure, quantity and number of nozzles and product characteristics such as viscosity) should be checked experimentally with the product to be sterilized;
- Lines which led steam and injector should be hygienic designed inside and out;
- During shutdown of the system the product may enter the line supplying steam through the injector nozzle for this should be able to clean the outside and inside. The injector should also be easy to be cleaned;

- No cleaning chemical residues should be remained in the line for delivering steam injector.

6.3 Containers for direct heating

- Containers for direct heating systems are: the mixing container and vacuum cooling container. They should be able to be cleaned and fully swell. This applies to all internal components and connections;
- All vacuum generating connections are critical as the vacuum created conditions for penetration of bacteria;
- Double seals increase security when the distance between them is flowing over with vapor or antimicrobial liquid.

7. Heat exchangers

There are three main types of heat exchangers: pipe, plate and ribbed walls. Each type is suitable for a specific application and a specific requirement. The general requirements are:

- Should be able to be cleaned completely, drain and available for inspection;
- All fluids used to clean the place must be compatible with the structural materials under conditions of use;
- Maintenance fluids should not be corrosive;
- If the active side of the heat exchanger should be drainable should be provided complete draining of all operating fluids;
- Slots and dead zones should be avoided on the product contact because they are difficult to clean;
- All connections in the sterile area of the system should be aseptic;
- Always should have two seals between the product and the flow of cooling/ heating medium. The space between them must be large enough;
- Changing of the seals should be performed according to the manufacturer instructions of the heat exchanger;
- If the products incorporating large amounts of soot, the channel of the product should not be too narrow to avoid congestion. For some products, increasing the speed helps control of the soot;
- To prevent corrosion, the design must prevent prom differences in the increase and decrease the volume;
- The flow of the product should be properly directed to avoid the retention of air in the system.

7.1 Tubular Heat Exchangers

- All welds should behave satisfactory quality according to the operating and cleaning conditions;
- Internal spaces should be easy to clean;
- Design should prevent the lamination of highly viscous products;
- If is impossible to reach high enough turbulence should be used sanitary mixing elements to ensure uniform temperature distribution at the end of the warm-up section;
- The design must prevent vibrations and resonances that weaken the screw connections.

7.2 Plate heat exchangers

- Plates manufacturer should inspect all plates for cracks;
- To reduce corrosion of the plates caused by mechanical damage of pulsations and vibrations should be used pads;
- Plates come in contact with the sterile product should be regularly inspected for mechanical damage. If information is given for the life of the plate, it must be replaced regularly;
- The surface roughness of the plates should be $Ra \leq 1\mu m$ defined by ISO 468:1982.

7.3 Ribbed walls heat exchangers

- Suitable for heating viscous products;
- All internal bearings must be hygienic and can be cleaned;
- Single seals are satisfactory for static applications. Dynamic seals must be capable of double wrapping steam or other antimicrobial liquid;
- The design must not allow the formation of airbags;
- The design should allow adequate cleaning of the round spot areas where the flow rate can be lower than the rest of the line.

7.4 Holding tube

- The restraint tube is critical element because it provides the necessary heat treatment of the product. It must be swell completely and easily cleanable;
- Must have a minimum number of connections that are suitable for aseptic work;
- If insulation is used, it should not be corrosive to the materials of the retaining section;
- Flow velocity and inclination to work to prevent the formation of air bags;
- The product entering the pipe does not contain non-condensable vapor, no change in the retention time;
- You can define a minimum retention time in the pipe of any product that will be produced;
- If soot is formed, should consider its impact on retention time;
- All sensors should be designed for aseptic work;

- When using direct heat to make allowance for the amount of steam that condenses in the product when determining the length of the retaining tube.

7.5 Buffer tanks

- Blind spots, sharp edges or narrow niches should be avoided;
- Materials should be compatible with cleaning product and chemical conditions of use and accessibility after installation;
- Buffer tanks should be able to drains completely, clean in place and sterilize with steam regardless of the attached equipment. If using a cooling jacket, it should be completely drained prior to sterilization. Combined input-output relation is preferred;
- It must be possible product in the buffer tank to remains sterile even if working installation or filling machine did not become sterile. This is achieved by aseptic barrier;
- Filters for sterile air are required to provide a sterile medium over the liquid. The filter must be protected from mechanical damage from excessive air speeds;
- If needed should be used two filters if only one is not enough.

7.6 Sequence of operation

Before sterilization of equipment

All surfaces in contact with the product must withstand operating temperature and time and have proper drainage. Sterilizing time and temperature depend on the configuration of the installation. Long not insulated pipes can lead to lower temperature sterilization than short and insulated pipes.

Production

Responsibility of the supplier of equipment is to ensure that the heat exchanger can achieve the desired temperature at all times. Level measuring system shall provide a minimum level always fluid in buffer to prevent the pump suck air.

Changing the direction of flow

Diverting valves are designed aseptically (often used to circulate hot water during sterilization installation). To prevent contamination of the aseptic tank or filling machine, there should be an aseptic barrier after deviator valve.

Cleaning

The chemicals must be compatible with construction materials at the temperature of use, and the concentrations and must be completely removed when rinsing with water.

8. Continuous or semi-continuous thermal processing of foods containing particulates

To ensure the microbiological safety of the sterilization process must meet the following requirements:

- Measuring and control equipment to ensure the maintenance of pressure, temperature and flow;
- Unacceptable variations in key parameters should lead to an automatic shutdown of the system;
- Equipment in sterile areas of the system should be easy to clean and impervious to bacteria;
- The process shall end when accumulating a certain amount of deposits (soot), which may worsen the microbiological parameters.

9. Hygienic packing of food products not requiring aseptic conditions

- All surfaces in contact with the product should be resistant to solvents and cleaning products used in temperature and are eligible for food contact;
- All surfaces in contact with the product should be cleaned;
- Attention must be paid to the swelling of the surfaces of packaging machines and to control condensation, which could be formed during the packaging.

9.1 Equipment for filling and dosing

- Should be able to be clean easily, preferably with a cleaning system in place;
- Should not include dead zones, cracks or areas with low speed cleaning fluid;
- Particular attention should be paid to the static and dynamic seals, as the temperature changes to which the equipment is exposed; it can lead to microbiological problems arising from differences in thermal expansion between the materials of construction.

9.2 Installation of Packaging Machine

- Packaging machines must be placed at the appropriate place and have free access to all around them. It should be set up not to keep dust and other foreign particles;
- Above it should not put any devices such as lamps and other pipelines;
- The space under the machine should allow better cleaning and effective inspection;
- The machine should not be placed near the traps (drains), because it would impede the inspection and cleaning;
- Equipment must be installed exactly where must be. Equipment legs must be firmly attached to the floor.

9.3 Product lines for packaging machines

Should be as short as possible and be suitably equipped and designed to allow easy cleaning and inspection.

9.4 Drainage systems

- You must ensure all areas where water or liquid spread during normal operations or where the floors are cleaned with hoses;
- Drains are maintained in good working conditions with unlimited access for inspection and cleaning.

9.5 Conveyors

- Must be constructed so as to prevent the retention of pollutants and to allow easy access for inspection and cleaning including inside bars and rollers;
- Dispensers and containers for lubricants storage shall be so designed as to prevent the entry of lubricants on surfaces in contact with the product or on the floor. They should be easily accessible for cleaning and inspection.
- Where lubricants are used on the surface of the strip, they must have an acceptable composition for contact with food;
- The lubrication system must be properly installed and maintained.

9.6 Types of additional devices to packaging machines to reduce microbial contamination during packaging

- Cover design by simply sealed cover to the tunnel with or without sterile air system;
- Decontamination air system, which may consist of a compressor and filter system or heating unit. Air can be used to pass in the tunnel structure or create a laminar air flow, so that the area of packaging machines to be protected;
- Devices for decontamination of packaging materials.

10. Aseptic packaging of food products

- All surfaces in contact with the product should be resistant to cleaning agents and temperatures at being used;
- Surface roughness of product contact should be Ra≤0,8 μm.

10.1 Equipment for filling and dosing

- Do no dead zones, cracks and areas with low speed cleaning fluid;
- All surfaces in contact with the product should be resistant to the product in terms of processing, generally should not be used stainless steel.

10.2 Contact with product

- The risk of infection can be reduced by reducing the time of contact with the air in open vessels, therefore the vessels should be sealed as soon as possible after filling;
- During the transition from the filling station to the sealing station, the product must be protected from air pollution;
- The air must be free of contamination.

10.3 Cleaning

- To reduce the difficulty of cleaning the area in contact with all the moving parts of the machine should be as far away from the zone located in contact with the product;
- When using the cleaning system, the moving parts are in motion during cleaning.

11. Storage and transport of packaging materials

- Protection (by the manufacturer of packaging material) of all packaging materials by wrapping in foil or put in boxes and others;
- Remove dust (through ionized air or filters);
- Ensure that surfaces in contact with product shell only touched with clean and protected hands;
- Should be provided areas for dry store of packaging materials.

11.1 Decontamination of packaging materials

When packing material must be sterilized, this can be achieved through the use of ultraviolet light, H_2O_2 and others.

11.2 Sterile air packaging supply system

Heating

The air sucked out of the superstructure of the machine in the super heater where it is heated to 400 °C, then cooled and fed into the packing machine.

Filtration

- Filters should be able to sterilize single or multiple;
- For single sterilizing filters should pay attention to the flow of air passing through the filter to be continuous even when the machine is not used;
- Sterilization of air filters can be done with chemicals or steam;
- Filters should be changed before it gets worse characteristics.

Closing of the container

- The construction of the container closure to ensure easy sealing;
- Temperature and pressure should be properly allocated sealing surfaces;
- All parameters of the sealing process (time, temperature and pressure) must be properly documented and monitored;

- If several packages are sealed with the same operation, the packages must be equally sealed;
- Contamination of the sealing surfaces of products (foam) should be avoided;
- The seal is properly positioned, especially in the case of heat seal machines /1/.

It is important to note that the legal framework of the European Union relating to food safety is missing a key element, namely the need to use equipment designed hygienic design. For example, Regulation 852/2004 on the hygiene of foodstuffs, Annex II, Chapter II, clause 1 (f) only implies a certain design techniques: surfaces (including surfaces of equipment) in areas where food is handled and in particular those coming in contact with food must be maintained in good condition and easy to clean and, where necessary, disinfected. This requires the use of smooth, easily cleanable, corrosion resistant and non-toxic materials, unless food business operator demonstrates to the supervisory authority that other materials used are appropriate.

Specific chapter of Directive 2006/42 EU machinery (Annex 1, Section 2), dedicated to the food industry provides more information, such as: "all surfaces that come in contact with foodstuffs or cosmetics or pharmaceutical products, other than surfaces disposable must:

- To be smooth, without edges or crevices which could harbor organic matter. These requirements apply to their compounds;
- Be designed and constructed in a way that reduces the projections, edges and recesses of assemblies to a minimum;
- Are easy to clean and disinfect where necessary after removing easily dismantled elements, inside surfaces must have curves with a radius sufficient to allow thorough cleaning "/2/.

Listeria monocytogenes may be present for months or years in the food business, forming a biofilm in hard to reach areas to clean the equipment. Therefore, it is essential to eliminate cleaning hard to reach areas in the premises and facilities. While the floors and sinks/ drains are covered by the regulations of the so-called hygiene package and are subject to supervision by regulatory authorities, and cleaning and disinfection of equipment, hygiene latest design is not addressed and therefore not controlled.

III. Conclusions

Hygiene design, along with other requirements of the program of best practices is a prerequisite for the production of safe food and beverages. At that time, EU legislation on food safety are not treated in depth and put relevant hygiene requirements designed equipment, but such requirements are entered in the guidelines of the European Group (EHEDG) for hygienic design and should be used by equipment manufacturers Food industry and taken into account in the selection of equipment by professionals in food businesses.

The managements of the EHEDG could be used as a communication tool between users and equipment suppliers that provide assurance that the purchased equipment or its individual components meet the hygienic design /4/.

Hygiene designed equipment is an important element that can be considered as a control measure in combination with operational effective cleaning and disinfection of equipment have synergistic action to eliminate or reduce to an acceptable level of different types of food contamination resulting from the production equipment.

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LIPID COMPOSITION OF SEEDS FROM FAM. *CUCURBITACEAE*

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Abstract. *The vegetable seed oils obtained of three species of genus Cucurbita (Family Cucurbitaceae) were investigated. The oil content in the seeds varied from 45.1 % to 51.2%. The biological active substances - fatty acids, phospholipids, sterols and tocopherols were studied. Fatty acid composition was established by gas liquid chromatography. Linoleic acid (35.6% - 50.8%) was the main component in the fatty acid fraction, followed by oleic acid (21.8% - 35.9%). Phospholipids were found to be 0.5% - 1.1 % in the raw oils. The contents of sterols and tocopherols were 0.5% - 0.7% and 233 - 420 mg/kg respectively. γ-Tocopherol (58.1% - 88.4%) predominated in the oils, followed by γ-tocotrienol (10.0-39.5%).*

Key Words: *Cucurbitaceae*, glyceride oils, fatty acids, phospholipids, sterols, tocopherols.

I. Introduction

Pumpkins belong to the genus *Cucurbita*, family *Cucurbitaceae*. The genus *Cucurbita* includes five species: *C. maxima*, *C. moschata*, *C. pepo*, *C. ficifolia* and *C. turbaniformis*. Pumpkin seeds are excellent source of oil (37.8-50.0%) and protein (25.2 – 37.0%). They are used for human consumption in many cultures all over the world. The pumpkin seed oil is used for industrial application and can contribute to healthy human diets. It has been used traditionally as medicine in many countries and it is applied in therapy of small disorders of the prostate gland and urinary bladder caused by hyperplasia. It has been reported that the pumpkin seed extracts have antidiabetic, antitumor, antibacterial, anticancer antimutagenic and antioxidant activities. It has also been found to have strong hypotriglyceridemic and serum cholesterol – lowering effects.

The oil content of pumpkin seeds is about 50% (45-60%). Pumpkin seed oil is dark green to red, with specific aroma and taste and has high content of free fatty acids [25]. The glyceride fraction contains about 80% unsaturated fatty acids, mainly linoleic (42.0 - 68.5%) and oleic (20-38%) acids. The content of saturated fatty acids was about 19%, mainly palmitic (13%) and stearic (6%) acids. The fatty acid composition varies depending on variety of areas in which the plants are grown, climate and state of ripeness. The pumpkin seed oils content 360-885mg/kg tocopherols and γ-tocopherol represented 96% of total tocopherols [7]. Sterols are 0.5% in the oil and Δ^7-sterols are the main components. The total phosphorus content is about 211μg/g in dry seeds [10]. Data on physicochemical characteristics of pumpkin seed oil show that it can be considered as a new and valuable source of edible oil. On the other hand the literature data on pumpkin seed oil of Bulgarian origin is very scarce.

The purpose of this work is to study the content and composition of the main biologically active components as fatty acids, phospholipids, sterols and tocopherols of the seed oils isolated from three species of the family *Cucurbitaceae*, grown in southern Bulgaria, on the forming qualities as food and pharmaceutical product.

II. Materials and methods

All solvents and reagents were of analytical grade from Merck (Darmstadt, Germany) and were used without additional purification.

Samples. The seeds of species *Cucurbita moschata*, *Cucurbita pepo* and *Cucurbita maxima*, fam. *Cucurbitaceae*, were grown and obtained from the region of South Bulgaria, crop 2012.

Isolation of glyceride oil and determination of oil content. The seeds (50g sample) were air-dried and ground to powder and the oil was extracted with n-hexane in *Soxhlet* apparatus for 8 h. The solvent was partly removed in rotary vacuum evaporator, the residue was transferred in pre-weight glass vessels and the rest of the solvent was removed under stream of nitrogen to a constant weight to determine the oil content [16].

Analysis of fatty acids. The fatty acid composition of oils was determined by gas chromatography (GC) after transmethylation of the respective sample with 2 % H_2SO_4 in absolute CH_3OH at 50^oC [15]. Fatty acid methyl esters (FAME) were purified by thin-layer chromatography (TLC) on 20x20 cm plates covered with 0.2 mm silica gel 60 G (Merck, Darmstadt, Germany) layer with mobile phase n-hexane : diethyl ether 97:3 (by volume). GC was performed on a HP 5890 (Hewlett Packard GmbH, Austria) gas chromatograph equipped with a 30 m x

0.25 mm capillary EC-Wax column and a flame ionization detector. The column temperature was programmed from 130°C (4 min), at 15°C/min to 240°C (5 min); injector and detector temperatures were kept at 250°C. Hydrogen was the carrier gas at a flow rate 0.8 ml/min; split was 1:50. Identification of fatty acids was performed by comparison of retention times with those of a standard mixture of fatty acids subjected to GC under identical experimental conditions [14]. The standard mixture of fatty acid methyl esters were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Iodine value (g I_2/100g fat) was calculated on the basis of fatty acid composition of the oil [6].

Analysis of sterols. Unsaponifiables were determined after saponification of the glyceride oil and extraction with hexane [18]. Quantification of sterols was carried out spectrophotometrically (at 597 nm), after isolation of sterols from other unsaponifiable matter by thin layer chromatography on Silica gel 60 G in the mobile phase diethyl ether: hexane (1:1 v/v) [19].

Analysis of tocopherols. Tocopherols were determined directly in the oil by high performance liquid chromatography (HPLC) on a "Merck-Hitachi" (Merck, Darmstadt, Germany) instrument equipped with 250 mm x 4 mm Nucleosil Si 50-5 column (Merck, Darmstadt, Germany) and fluorescent detector "Merck-Hitachi" F 1000. The operating conditions were as follows: mobile phase of n-hexane : dioxan 96:4 (by volume), flow rate 1.0 ml/min, excitation 295 nm, emission 330 nm [17]. 20 μl 1% solution of oil in hexane were injected. Tocopherols were identified by comparing the retention times with those of authentic individual tocopherols. Reference tocopherol homologues were purchased from Merck (Darmstadt, Germany). The tocopherol content was calculated on the basis of tocopherol peak areas in the sample versus tocopherol peak area of standard α-tocopherol solution.

Analysis of phospholipids. Another part (100g) of air-dried seeds was subjected to Folch extraction [9] and polar lipids were isolated from the total lipids by column chromatography. The phospholipids classes were isolated by a variety of the two-dimensional TLC on 20 x 20 cm glass plates with 0.2 mm Silica gel 60 G layer impregnated with aqueous $(NH_4)_2SO_4$ (1g in 100 ml water). In the first direction the plate was developed with chloroform: methanol: ammonia, 65: 25: 5 (by volume) and in the second – with chloroform: acetone: methanol: acetic acid: water, 50:20:10:10:5 (by volume). The individual phospholipids were detected and identified by spraying with specific reagents: Dragendorff test (detection of choline-containing phospholipids), Ninhydrin spray (for phospholipids with free amino groups) and Shiff's reagent (for inositol containing phospholipids). Additional identification was performed by comparing the respective R_f values with those of authentic commercial standards subjected to silica gel TLC under identical experimental conditions. The quantification was carried out spectrophotometrically against a standard curve by measuring the phosphorous content at 700 nm after scrapping the respective phospholipid spot and mineralization of the substance with a mixture of perchloric acid and sulphuric acid, 1:1 by volume [13].

III. Results and discussion

Data about content of oil in the three species pumpkin seeds and biologically active substances in the oil and the seeds are provided in Table 1.

The oil content of pumpkin seeds in investigated species was ranging from 45.1% to 51.5% and it was similar to the quantities reported earlier. A number of authors indicate that pumpkin seeds of Cucurbita pepo contain about 50% (45-60%) oil [25], 50.3-51.0% oil [11,12], 43.4-47.2% oil [20] and 41.59% oil [7].

Table 1. *Oil content and main biologically active substances in the three species of pumpkin seed oils*

Compounds	**Pumpkin**			**Pumpkin oil trademark**
	Cucurbita moschata	*Cucurbita pepo*	*Cucurbita maxima*	(*Balcho*)
Oil in the seeds , % wt	45.1	46,8	51.5	-
Sterols				
- in the oil, % wt	0.7	0.5	0.6	0.6
- in the seeds, % wt	0.3	0.2	0.3	-
Phospholipids				
- in the oil, % wt	0.5	1.1	1.0	0.5
- in the seeds, % wt	0.2	0.5	0.5	-
Tocopherols				
- in the oil, mg/kg	417	292	233	1077
- in the seeds, mg/kg	188	137	120	-
Iodine value, g J_2/100g	99	114	97	102
Oxidative stability, h	23.0	23.2	22.2	26.3

The oil content in the seeds of *Cucurbita moschata* ranges from 43.0 – 50.81% [2, 4, 21]. The seeds of *Cucurbita maxima* contains 50.81 – 52.43% oil [2, 21]. There are data for lower oil content in the seeds of the *Cucurbita maxima* – 10.9-30.9% [3, 24].

The total content of phospholipids, sterols and tocopherols is close to data reported for other vegetable oils [8]. The content of phospholipids in the studied seeds of pumpkin varieties was lower than that determined by Tri Joko Raharjo et al. (1.27% total phospholipids in the pumpkin seeds) [23]. The content of tocopherols was found to be in *Cucurbita moschata* and *Cucurbita pepo* seeds oil (417-420 mg/kg), while *Cucurbita maxima* seed oil has 2 times lower quantities. According Ardabili et al. [7] and Gemrot et al. [11] the total tocopherol content in oil of pumpkin seeds (*Cucurbita pepo*) was 882.65 mg/kg and in the seeds was 107 mg/100g. Probably the commercial pumpkin oil contains additional quantity tocopherols as antioxidants because it contains 1077 mg/kg. The amounts of sterols and phospholipids in the investigated oils of the pumpkin varieties and that from the trade mark *Balcho* are of the same order. The iodine number, which is a measure of the degree of unsaturation of fatty acids in vegetable oils is relatively high (IV = 97÷ 114 gJ_2/100g), as a result of the higher content of essential unsaturated acids. This value is close to data reported by Ardabili (104.4) [7] and Al Khalifa (111.5) [4] for *Cucurbita pepo*, 83.8 and 113.5 for *Cucurbita moschata* [2, 4], 100.6 and 105 for *Cucurbita maxima* [1, 3]. The iodine values are close to those of rapeseed (94-120 gJ_2/100g) and canola oil (98 gJ_2/100g) [8, 22]. Iodine value is an indirect indicator for the oxidative stability of the oils. Nearby values of iodine number of oils were tested in accordance with uniform oxidative stability (22.2h – 23.2h).

Fatty acid composition is one of the main parameters which are used for characterization of the oils in terms of their nutritional value, and with regard to their oxidative stability during storage. It can be varied in dependence of the climatic conditions where the species were grown [5]. The fatty acid composition of the investigated species of pumpkin seed oils are presented in Table 2.

Table 2. *Fatty acid composition of pumpkin seed oils* *

Fatty acids, (% wt of total)**	**Pumpkin**			**Pumpkin oil trademark**
	Cucurbita moschata	*Cucurbita pepo*	*Cucurbita maxima*	(*Balcho*)
$C_{8:0}$	0.2	0.2	0.2	-
$C_{10:0}$	-	0.3	-	-
$C_{12:0}$	-	0.3	-	0,1
$C_{14:0}$	0.2	0.4	0.4	0,3
$C_{16:0}$	25.7	17.7	19.7	16,6
$C_{16:1}$	0.1	0.2	0.2	0,2
$C_{17:0}$	0.1	0.1	0.1	0,1
$C_{18:0}$	7.7	5.6	7.5	6,5
$C_{18:1}$	21.8	24.0	35.9	39,3
$C_{18:2}$	43.8	50.8	35.6	36,3
$C_{18:3}$	0.2	0.2	0.2	0,4
$C_{20:0}$	0.1	0.1	0.1	0,1
$C_{20:1}$	0.1	0.1	0.1	0,1

*Mean of three separate determinations

** *$C_{8:0}$- Caprylic acid; $C_{10:0}$- Capric acid;$C_{12:0}$- Lauric acid; $C_{14:0}$- Myristic acid; $C_{16:0}$- Palmitic acid; $C_{16:1}$- Palmitoleic acid; $C_{17:0}$- Margaric acid, $C_{18:0}$- Stearic acid; $C_{18:1}$-Oleic acid; $C_{18:2}$- Linoleic acid; $C_{18:3}$- Linolenic acid; $C_{20:0}$– Arachidic acid; $C_{20:1}$- Eicosenoic acid (gadoleic)*

Thirteen fatty acids were detected. The major fatty acids were linoleic acid ($C_{18:2}$) which content varies from 35.6% (*Cucurbita maxima*) to 50.8% (*Cucurbita pepo*) and oleic acid ($C_{18:1}$) which content varies from 21.8% (*Cucurbita moschata*) to 35.9% (*Cucurbita maxima*). The presence of high amounts of the essential linoleic acid suggests that the pumpkin seed oil is highly nutritious. Palmitic acid ($C_{16:0}$) predominant in the saturated fatty acid fraction - from 17.7% in *Cucurbita pepo* to 25.7% in *Cucurbita moschata*, followed by stearic acid ($C_{18:0}$) - from 5.6% in *Cucurbita pepo* to 7.7% in *Cucurbita moschata*. Linolenic acid was identified in negligible amount (0.2%). The oils of *Cucurbita pepo* and *Cucurbita moschata* contain similar amounts of oleic and linoleic acids, but *Cucurbita maxima* seeds contain lower quantity of linoleic acid and higher quantity of oleic acid. Fatty acid composition of commercial pumpkin oil (*Balcho*) is close to that of the oil from the seeds of variety *Cucurbita maxima* – contained similar amounts of linoleic acids (*C. maxima* - 35.6%, *Balcho* - 36.3%) and oleic acid (*C. maxima* - 35.9%, *Balcho* - 39.3%). The fatty acid composition of pumpkin oil from the studied species

is similar to this reported by previous studies - linoleic acid (35.72 - 56.60%), oleic acid (14.83 - 38.10%), palmitic acid (10.68 - 16.41%) and stearic acid (4.67 - 11.14%) [2, 3, 4, 7, 10, 11, 21, 24].

Figure 1 shows that the unsaturated acids predominate in the pumpkin oils (*Cucurbita moschata*: 66.0 %; *Cucurbita maxima*: 72.0% and *Cucurbita pepo*: 75.3%). Polyunsaturated fatty acids predominate in the *Cucurbita pepo* oil (51.0%) and *Cucurbita moschata* (44.0%), while in the *Cucurbita maxima* and trademark *Balcho* pumpkin oil monounsaturated and polyunsaturated are in the same amount. The total saturated fatty acid content range from 24.7% (*Cucurbita pepo*) to 34.0% (*Cucurbita moschata*). These results confirm data of Al-Khalifa (SFA – 19.1-19.7%, MUFA – 26.64-53.33% and PUFA – 36.1-44.0%) [4], Alfawaz (SFA – 27.73%, MUFA – 19.06% and PUFA – 53.97%) [3], Achu (SFA – 21.7-30.2%, MUFA – 19.4-25.3% and PUFA – 49.38-52.63%) [2], Mi Young Kim (SFA – 17.47-20.11%, MUFA – 14.9-32.4% and PUFA – 35.72-56.84%) [21].

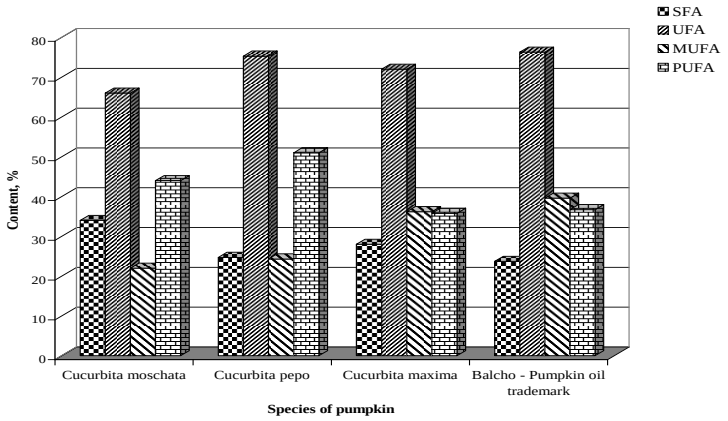

Figure 1. *Saturated (SFA) and unsaturated (MUFA - monounsaturated, PUFA - polyunsaturated) fatty acids in oils from seeds of various species pumpkin*

Tocopherol composition was determined directly to the oils by high pressure liquid-liquid chromatography with fluorescence detection and the obtained results are presented in Table 3.

Table 3. *Tocopherol composition of oils from the seeds of a different pumpkin species* *

Tocopherols, (% wt of total)	**Pumpkin**			**Pumpkin oil trademark**
	Cucurbita moschata	*Cucurbita pepo*	*Cucurbita maxima*	(*Balcho*)
α - tocopherol	1.6	7.1	2.4	4.8
γ - tocopherol	88.4	71.7	58.1	91.3
γ - tocotrienol	10.0	21.2	39.5	1.9
δ - tocopherol	-	3.3	-	2.0

γ - Tocopherol (58.1% to 88.4%) was dominated component in *Cucurbita pepo*, *Cucurbita moschata* and *Cucurbita maxima* pumpkin seed oils followed by γ-tocotrienol (10.0 - 39.5%). In the oil of *Cucurbita pepo* was found a higher content of α-tocopherol (7.1%) than the other two varieties (1.6-2.4%). δ - Tocopherol was found only in the trademark pumpkin oil (2.0%). Mi Young Kim et al. [21] reported that the seeds of *Cucurbita pepo* and *Cucurbita moschata* have significantly higher quantity of γ - tocopherol (61.65-66.85mg/kg in raw weight) than in *Cucurbita maxima* seeds (28.70mg/kg raw weight). It was found that the content of γ - tocopherol in oil from seeds of *Cucurbita pepo* and *Cucurbita moschata* was 2-3 times higher than α-tocopherol. In another reports in the oil of twelve pumpkin cultivars (*Cucurbita maxima*) α-tocopherol ranged from 27.1 to 75.1 μg/g in the oil, γ - tocopherol from 74.9 to 492.8 μg/g and δ - tocopherol from 35.3 to 1109.7 μg/g [24].

Tocopherol composition of trademark pumpkin oil has a different composition than the studied raw pumpkin species – it was established 91.3% content of γ - tocopherol. This composition is close to data obtained by Gemrot [11] for *Cucurbita pepo* pumpkin oils, where γ - tocopherol represented 96% of total tocopherols.

Results show that the tocopherol composition of pumpkin seed oil is significantly different than the composition of other vegetable oils, where α-tocopherol dominates, but is similar to the composition of soybean, corn and sesame oils, which are rich mainly in γ-tocopherol [8, 22].

IV. Conclusion

The investigated pumpkin *Cucurbita moschata*, *Cucurbita pepo* and *Cucurbita maxima* seeds from Bulgarian origin contain high quantities of glyceride oil (45.1% - 51.5%) which is rich in polyunsaturated fatty acids especially linoleic acid and have low amounts of saturated fatty acids. They have a similar lipid composition as the other commercial oils and higher oxidative stability than other seed oils. This makes them very valuable source of edible oil.

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THE CHANGES OF PHOSPHOLIPID AND FATTY ACID COMPOSITION DURING DEVELOPMENT OF FLAX SEEDS

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Abstract. *The changes of content and composition of flax seed phospholipids and fatty acids, during vegetation in period April - July were investigated. The quantity of glyceride oil increased from 17.4% to 32.7% between 21 - 35 day after flowering (DAF). The percentage of phospholipids was highest in the first stage of development (1,7% vs 1,2 % in the last period). In triacylglycerol fraction the content of oleic acid decreased from 38,3% to 32,2% at the expense of higher quantity of linolenic acid (from 29,4% to 34,2%). In phospholipids higher content of monounsaturated oleic acid was detected – from 56,9% at 21 DAF to 51,8% at 35 DAF. Higher quantity of saturated and monounsaturated acids in phospholipid than in triacylglycerol fraction was established.*

Keywords: flaxseed, vegetation, phospholipids, triacylglycerols, fatty acids

I. Introduction

Flax (*Linum usitatissimum* L.) is one of the oldest and multi–purpose oilseed crops cultivated in Europe and Asia (Beltagi, 2007; Berglund, 2002). The seeds are rich in carbohydrates, proteins and dietary fibers (Herchi *et al.*, 2012). Increasing interest in the glyceride oil has been inspired by dietary recommendations for beneficial nutritional effect, result of high content of polyunsaturated fatty acids including primarily linoleic acid and α-linolenic belonging to omega – 3 (Piłat and Zadernowski, 2010). Unsaturated fatty acids, mainly linolenic and linoleic are the major components (85%) in triacylglycerols. Gunstone, (2002) reported that linolenic was about 47,4%, followed by linoleic – 24,1% and oleic – 19,0% and level of tocopherols – 440 – 558 mg/kg in the oil. According Choo *et al.* (2007), linolenic acid predominated in linseed oil (51,8 – 60,4%), followed by linoleic and oleic acid. Bozan and Temelli, (2008); Choo *et al.*, (2007), reported about 70.5% linolenic acid in the oil while saturated fatty acids ranged from 9,0 to 12,0%.

The nutritional value of the oil besides essential fatty acids depends on the content and composition of other biologically active substances as sterols, phospholipids and tocopherols. The phospholipids have an important role in food industry as good emulsifiers and stabilizer of emulsions for improve of food texture, for reduction of plasma lipoprotein cholesterol (Wilson *et al.*, 1998).

It is of interest the accumulation of fatty acids in the triacylglycerols and in the phospholipids as well as phospholipids in the oils of the seeds in connection to prognosticate the composition of them during development. Oomah and Sifter, 2009 reported about 2,0 – 3,0% phospholipids in the oil. According Herchi *et al.* (2011), in phospholipid fraction the main components were found to be phosphatidylethanolamine (PEA) – 27,0 – 40,0%, lysophosphatidylcholine (LPC) – 8,0 – 21,0%, phosphatidylinositol (PI) – 29,0 – 32,0%, phosphatidylcholine (PC) – 7,0 – 18,0, diphosphatidylglycerol (DPG) - (1,0 – 4,0%). Palmitic and stearic acid were established as the predominant constituents in phospholipid fraction.

The aim of this study was to determine the changes of content of glyceride oil in the seeds and content as well as the changes of individual composition of triacylglycerols and phospholipids during vegetation of flax seeds.

II. Materials and methods

***Reagents and standards*.** All solvents and reagents (reference fatty acids, phospholipids) are with analytical grade provided from Merck (Darmstadt, Germany). Thin layer chromatography plates (TLC), (0,2 mm, 20 x 20 cm) for purification of fatty acid methyl esters and phospholipids were prepared in laboratory using silica gel 60 G (Merck, Darmstadt, Germany).

***Samples*:** The flax seeds were grown on plantation in Plovdiv region, Bulgaria in April – July, 2012. The samples were analyzed in 21, 28 and 35 day after flowering (DAF).

Oil content. The seeds (20g sample) were air-dried (10% humidity) and oil was extracted with hexane in a Soxhlet apparatus for 8h (ISO 659:2009). After extraction the solvent was removed in a rotary vacuum evaporator and oil was determined by weight.

Phospholipid composition. Another part (10g) of air-dried seeds were subjected to Folch extraction according to Christie, (2003). Polar lipids were isolated from the total lipids by column chromatography. Briefly, the sample (100 mg) was applied on a 40 cm x 2 cm glass column packed with Silica gel Unisil 100-200 mesh (Clarkson Chemicals Co., USA) and eluated in sequence with chloroform (for neutral lipids, sterols and sterol esters), acetone (sterol glycosides) and with methanol to isolate phospholipids. The phospholipid classes were isolated by a variety of the two - dimensional thin - layer chromatography (TLC) on 20 x 20 cm glass plates with 0.2 mm Silica gel 60 G layer (Merck) impregnated with aqueous $(NH_4)_2SO_4$ (1 g in 100 ml water). In the first direction the plate was developed with chloroform : methanol : ammonia, 65 : 25 : 5 and in the second - chloroform : methanol : ammonia : acetic acid : water, 50 : 20 : 10 : 10 : 5 (by volume). The individual phospholipids were detected and identified by spraying with specific reagents according to Christie, (2003): Dragendorff test (detection of choline-containing phospholipids); Ninhydrin spray (for phospholipids with free amino groups), and Shiff's reagent (for inositol containing phospholipids). Additional identification was performed by comparing the respective R_f values with those of authentic commercial standards subjected to Silica gel 60 G TLC under identical experimental conditions. The quantification was carried out spectrophotometrically against a standard curve by measuring the phosphorous content at 700 nm after scrapping the respective phospholipid spot and mineralization of the substance with a mixture of perchloric acid and sulphuric acid, 1:1 by volume. Etalon – 10 mkl/cm^3 water solution of KH_2PO_4 as P (ISO 10540, 2003). Content of phospholipids in the sample – 1-125 mkg/kg as P.

Fatty acid composition. Phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine were isolated from other polar lipids by preparative thin-layer chromatography on 20 x 20 cm glass plates with 0.2 mm Silica gel 60 G layer (Merck) impregnated with aqueous $(NH_4)_2SO_4$ (1 g in 100 ml water) with mobile phase chloroform : methanol : ammonia : acetic acid : water, 50 : 20 : 10 : 10 : 5 (by volume). Fatty acids of triacylglycerols and of individual phospholipids were determined by gas chromatography (GC) of fatty acid methyl esters (FAME) (ISO 5508, 1990). FAME were prepared by pre-esterification with sulfuric acid in methanol as catalyst (Christie, 2003). The FAME was purified by TLC on silica gel 60 G with mobile phase hexane : acetone 10 : 8 (by volume). The operating conditions were as follows: GC HP 5890 A equipped with 60 m x 0,25 mm x 0,25 μm DB-23 column and FID detector. The temperature gradient was from 130 ^{0}C for 1.0 min, 130-170 ^{0}C at 6.5^{0}C/min, 170-215 ^{0}C at 30^{0}C/min, 215^{0}C for 9 min, 215-230 ^{0}C at 40 ^{0}C/min to 230 ^{0}C for 1 min. Hydrogen was the carrier gas, split 100:1. The temperature of detector and injector was kept at 270 ^{0}C. Identification of fatty acids was performed by comparison with standard mixture.

Statistics:

All analyses were made in triplicate. The level of significance was set at $p<0.05$.

III. Results and discussion

Data about changes of oil content in the seeds and individual composition of phospholipid fraction are presented in Table 1.

Table 1. *Changes of oil content in the seeds and individual composition of phospholipid fraction*

	Day after flowering (DAF)		
Components	***21 DAF***	***28 DAF***	***35 DAF***
1. Content of oil in the seeds, %	17,4	29,1	32,7
2. Content of phospholipids in the oil, %	1,7	1,3	1,2
Phospholipids, %			
3. Phosphatidylinositol	32,8	32,7	32,1
4. Phosphatidylcholine	46,6	48,2	52,3
5. Phosphatidylethanolamine	12,0	11,2	9,2
6. Phosphatidylserine	1,5	1,6	1,0
7. Sphingomieline	2,2	3,1	1,9
8. Phosphatidic acids	2,5	3,5	3,4
9. Diphosphatidylglycerol	1,6	-	-

Total content of oil in the seeds is increasing and phospholipids are decreasing gradually in all investigated period. Data about individual composition leads to the conclusion that during the development there is mainly a synthesis of phosphatidylcholine (from 46,6 to 52,3%), at the expense of phosphatidylethanolamine. The level of phosphatidylinositol was relatively constant in all period of vegetation. The other classes of phospholipids were found to be in negligible quantities and change little during the growing process.

The changes of fatty acid composition of triacylglycerols and individual phospholipids during development are presented in Table 2.

Table 2. *Changes of fatty acid composition of triacylglycerols and individual phospholipids during development*

Fatty	21 DAF				28 DAF				35 DAF			
acids, %	***PI***	***PEA***	***PC***	***TAG***	***PI***	***PEA***	***PC***	***TAG***	***PI***	***PEA***	***PC***	***TAG***
$C_{12:0}$	0,2	0,3	0,6	-	0,1	0,3	0,1	-	0,3	0,3	0,1	0,1
$C_{14:0}$	0,8	1,0	1,2	-	0,4	0,7	0,4	-	0,5	0,8	0,4	2.5
$C_{15:0}$	0,4	0,4	0,4	-	0,3	0,4	0,2	-	0,3	0,4	0,2	-
$C_{16:0}$	35,1	23,2	20,4	8,8	31,8	22,7	14,6	7,2	30,6	20,8	13,5	9,8
$C_{17:0}$	0,4	0,3	0,3	-	0,4	0,3	0,2	-	0,8	2,8	0,2	0,1
$C_{18:0}$	10,2	6,5	8,5	8,3	9,0	5,0	5,8	7.1	8,6	5,7	4,8	4,1
$C_{18:1}$	30,4	34,1	56,9	38,3	28,9	33,4	51,4	37,3	29,4	30,9	51,8	32,3
$C_{18:2}$	11,7	21,0	7,4	14,6	16,7	24,6	20,1	16,5	18,0	25.5	22,4	16,5
$C_{18:3}$	10,4	12,2	3,7	29,4	11,8	12,0	6,6	31,6	11,5	12,8	6,3	34,2
$C_{20:0}$	-	-	0,2	0,3	-	-	-	0,3	-	-	-	0,4
$C_{20:1}$	0,3	0,6	0,3	0,3	0,3	0,5	0,4	-	-	-	0,3	-
$C_{22:0}$	0,1	0,4	0,1	-	0,3	0,1	0.2	-	-	-	-	-
SFA	47,2	32,1	31,7	17,4	42,3	29,5	21,5	14,6	41,1	30,8	19,2	17,0
MUFA	30,7	34,7	57,2	38,6	29,2	33,9	51,8	37,3	29,4	30,9	52.1	32,3
PUFA	22,1	33,2	11,1	44,0	28,5	36,6	26,7	48,1	29,5	38,3	28,7	50,7

Twelve fatty acids were detected in the phospholipid and triacylglycerol fraction of the oils. Quantitative fatty acid composition of triacylglycerols and individual phospholipid classes was similar but the quantitative profile is different. Oleic and linolenic acids predominated in the triacylglycerols during all period of development while the main components in phospholipids were palmitic, stearic and oleic acids. The level of saturated fatty acids, mainly palmitic and stearic acid decreased gradually during vegetation in all individual phospholipids at the expense of the increase of the linoleic acid. Significant differences between separate phospholipid classes were found to be too. Highest content of saturated fatty acids was established in phosphatidylinositol (47,2% for 21 DAF, 42,3% for 28 DAF and 41,1% for 35 DAF), followed by phosphatidylethanolamine and at least phosphatidylcholine. Very high content of oleic acid (57,2%; 51,8% and 52,1% respectively) was detected in PC during vegetation. Generally the composition of PC was found to be the closest to this of triacylglycerols, followed by phosphatidylethanolamine and phosphatidylinositol.

The changes observed in the fatty acid composition of phospholipids can be also explained with theory similarly to the formation of fatty acid composition of triacyglycerols (Ivanov, 1991; Garces and Mancha, 1989).

Since phospholipids are synthesized mainly during the first days of the growing process, before the biosynthesis of triacyglycerols starts, most likely they only go through the first fermentation system which synthesizes mostly the oleic acid and saturated fatty acids that combine in phospholipids. When the second fermentation system starts acting, most phospholipids are already synthesized and their biosynthesis process starts running at a lower activity rate. This leads to a slight increase in the correlation unsaturated : saturated fatty acids.

Individual phospholipids are synthesized in the following order : phosphatidic acids, phosphatidylinositol, phosphatidyletanolamine, phosphatidylcholine and only then triacyglycerols. Therefore, the phosphatidylcholine which is synthesized just before the triacyglycerols has fatty acid composition closest to theirs.

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HEAT WASTE RECOVERY FROM EXHAUST GASES OF HEAVY FUEL OIL BOILER BY USING HEAT EXCHANGERS WITH HEAT TUBES

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Abstract. *Commonly used in engineering practice are the steam boilers operating with heavy fuel oil. Therefore the efforts are focused on the improvement of their effectiveness. One way is to be utilized the waste heat with the exhaust gases and subsequent use of this heat for heating the air needed for burning or heating the feeding water. The abovementioned can be achieved through the installation of heat exchangers cooling the gas to a temperature higher than the dew point providing non corrosion operation of the system. In current paper are calculated and presented the optimal values of temperature of the air at the inlet of the air heater as well as at the outlet at different content of sulfur in the fuel. In addition it is proposed a method for heat recovery of exhaust gases of boiler Viessmann vitomax 200 type using air heater with thermosyphon heat pipe type.*

Key Words: heat exchanger, heat pipes, waste heat recovery, exhaust gases

I. Introduction

In persuasion of a higher level of energy efficiency of the industrial steam generators burning heavy fuel oil, various companies offer technical solutions related with decreasing of the heat losses with exhaust flue gases. Part of the technical solutions is to utilize the heat waste by reducing the gas temperature and subsequent utilization of the heat for heating the air needed for burning or heating of the feeding water. Achieving this task can be done through different types of heat exchanging devices, part of which cool the gases minimally [1, 3, 4, 7, 8] without reaching the dew point and the others utilize the latent heat of condensation [5]. One of the most important requirements for the realization of the utilization mechanism is the evaluation of the criteria for corrosion safety during the operation of air heaters for heavy fuel oil steam generators.

Low-temperature corrosion means the destruction of the metal surfaces of the air heaters, located in areas with the lowest operating temperatures on the path of the gases and air. The corrosion processes are consequence of various sulfur compounds, which are part of the chemical composition of the burning fuel. Fuel burning is accompanied with the following chemical reactions:

$$S + O_2 \rightarrow SO_2 \quad (1)$$

$$2SO_2 + O_2 \rightarrow 2SO_3 \quad (2)$$

However, along with these reactions, another reaction occurs with the transportation of the gas along the tract:

$$SO_3 + H_2O \rightarrow H_2SO_4 \quad (3)$$

During the process of the exhaust gases cooling in a certain moment, the vapours of the sulfuric acid begin to condense on the heating surfaces of the air heaters. The corresponding temperature of this process is called "thermodynamic dew point". Reaching this temperature is the beginning of oxidation-recovering processes, which ultimately lead to the gradual destruction of the metal surface of the air heater. Theoretically, there are two approaches in the protection process against corrosion. Firstly, it is well known that the rate of sulfuric-acid corrosion has a maximum value below the "thermodynamic dew point", and when this value slightly decreases the corrosion is reduced. Such assumption, however, is somewhat conditional, because after passing the maximum corrosion rate the corrosion velocity decreases slightly, but then again begins to grow. The approach related with the keeping of a constant and higher than the dew point temperature of the exhaust gases after the boiler is economically unjustified, due to lower values of the energy efficiency.

Experts from "Technology of the water and fuels" department of the Moscow Power Engineering Institute (Technical University) suggest a certain value for such temperature of the exhaust gases after the boiler, where the low-temperature corrosion does not exceed some acceptable value.

II. Study of the corrosion rate based on the fuel composition

The presented below calculation are accomplished by the means of the web based "on-line" calculator developed by the Moscow Power Engineering Institute [6], where the optimum temperature of the exhaust gases is defined, and the admissible corrosion rate does not exceeded 0,2 mm/year. In these calculations are set the characteristics of different heavy fuel oil with different content of sulfur, commonly used as fuel for industrial steam generators.

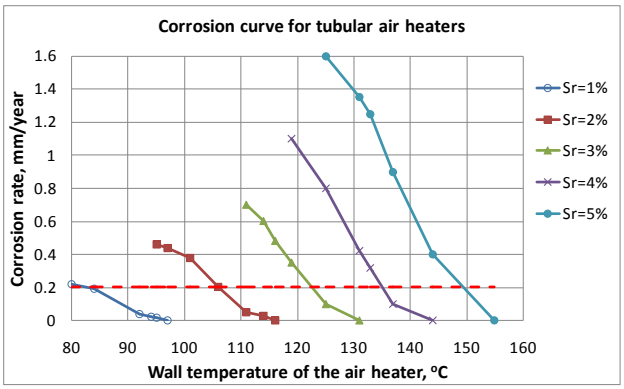

Figure 1. *The relation of the corrosion rate as a function of the surface temperature of the air heater tubes of a heavy fuel oil industrial steam generator Viessmann vitomax 200 type.*

On Figure 1 are presented the maximum values for the surface temperature of the air heaters at selected rate of corrosion. The same authors [6] are developed web base calculator for determination of the optimal temperature for the air at the inlet of air heater and the optimum temperature of the exhaust flue gases. The calculations for the steam generator type Viessmann vitomax 200 operating with heavy fuel oil, and with a steam production rate of 16 t / h are presented in the Table 1.

Table 1. *Dew point temperature and optimum temperatures for air heaters in accordance with [6].*

LHV=41 GJ/t; asses air rate in chamber 1.15	Sulphur content in heavy fuel oil, S^r, %				
Temperature difference in "cold" side of airheater =70^{0}C	S^r=1%	S^r=2%	S^r=3%	S^r=4%	S^r=5%
Dew point temperature, ^{0}C	96.9	116.3	167.3	143.7	154.8
Optimum air heater input temperature, ^{0}C	57.9	81.0	97.3	105.6	122.4
Optimum exhaust flue gas temperature, ^{0}C	127.9	151.0	167.3	175.6	192.4

The chemical analysis of the heavy fuel oil indicates that the sulfur content in the mixture of the fuel does not exceed 2%. Therefore, the accepted approach for utilization of heat waste from gases by installation of pipe air heater will increase the boiler efficiency with 2,9%, as far as a 0,2 mm/year corrosion rate is accepted. Considering that the pipe air heaters are usually made by tubes with wall thickness from 1,6 up to 2 mm determines the lifetime of the pipe air heater of around 2 up to 3 years. Such an investment is justified, but is also too risky.

III. Method for heat recovery of the exhaust gases of Viessmann vitomax 200 steam generator

An alternative method is proposed for heat recovery from the exhaust gases for a heavy fuel oil boiler type Viessmann vitomax 200. The method for heat recovery suggest the use of air heater with heat pipes of thermosyphon type [1, 3] being used the experience of the implemented in the period between 2003 and 2005y. four air heaters in Thermal Power Station "Maritsa Iztok - 2" for steam generators type PK-38 burning the sulfurous lignite with high moisture content [1,3].

A comparative analysis between standard horizontal-tube air heater and the proposed for implementation in Tiger Corporation town Pirot (Serbia) air heater with heat pipes (AH-HP) with regard to the safety of pipe corrosion surfaces is made. The study is focused on the final path of the flue gases where the pipes are exposed to the most severe modes. The only criterion to prevent corrosion processes on the surface of the pipes is the wall surface temperature to be kept higher than the dew point temperature of the exhaust gases in all modes of operation of the boilers [3, 8].

The theoretical dew point with a sufficient accuracy can be determined according the relation [8]:

$$t_{op}^T = \Delta t_{op}^T + t_{H_2O} \qquad (4)$$

where t_{op}^T [^oC] is the theoretical temperature difference between the acid temperature of dew point of gases and temperature of condensation of the water vapour; t_{H_2O} [^oC] – temperature of dew point of pure water vapour [8];

$$t_{op}^T = \frac{200\sqrt[3]{S_{np}^r}}{1,25^{a_{отн}.A_{np}^r}}, \ ^oC, \qquad (5)$$

where $a_{отн}$ - share of the carried away with the ashes; S_{np}^r and A_{np}^r - reduced contents of sulfur and ash relative to 1000 KJ / kg from the thermal value of heavy fuel oil [2].

In Table 2 are presented the results of the calculated "thermodynamic dew points" using empirical equations with different sulfur content in the elemental composition of the heavy fuel oil, calculated according the Normative Method (II-1) [8].

Table 2. *Dew point temperature for different sulphur content of heavy fuel oil.*

LHV=41 GJ/t; asses air rate in chamber 1.15	Sulphur content in heavy fuel oil, S^r, %				
	S^r=1%	S^r=2%	S^r=3%	S^r=4%	S^r=5%
Dew point temperature, ^{0}C	108.8	124.4	135.4	144.1	151.5

According to the presented relations (4), (5) for the selected fuel ($S^r = 2\%$; $LHV = 41\ GJ/t$) for the steam generator Viessmann vitomax 200 type and Table 2, the theoretical dew point occurs: $t_{dp}^T = 124.4\ ^oC$.

Full termination of the low temperature corrosion for the air heaters is ensured, if the wall temperature of the coldest area is higher than the temperature of the dew point with no less than $5 \div 10\ ^oC$ (minimum temperatures refer to the minimum loads) [8].
Similar calculations are made for the surface temperatures of the wall for a heat pipe air heater (AH – HP). The average surface temperature of the tube is determined (t_w^{av}), as well as the minimum possible surface temperature of the wall ($t_w^{\min}$) (concerning the final path of exhaust gases). The temperatures are calculated according to the well known relations in literature [8]:

$$t_w^{av} = \frac{\theta_1 + \theta_2}{2} - \frac{B^{calc}.Q}{H_{gas}}.\left(\frac{1}{\alpha_1} + \varepsilon\right), \qquad (6)$$

$$t_w^{\min} = \theta'' - \frac{B^{calc}.Q}{H_{gas}}\left(\frac{1}{\alpha_1} + \varepsilon\right), \qquad (7)$$

For the set of heat tubes the value of surface temperature is shown in Table 3. The illustration of this relationship is presented in Figure 2.

Table 3. *Dew point temperature for different sulphur content of heavy fuel oil.*

Parameter	Temperature of the incoming air, $t_{in.air,}$ ^{o}C				
	0 °C	20 °C	30 °C	40 °C	50 °C
Top row wall temperature, $t^t_{w,}$ °C	179.4	182.8	185.6	188.9	193.1
Middle row wall temperature, $t^m_{w,}$ °C	150.8	154.1	156.8	160.2	164.1
Lowest row wall temperature, $t^l_{w,}$ °C	122.2	125.4	128	131.5	135.1

The analysis which can be made based on Figure 2 is that almost all presented modes for the set of tubes of VP-TT will work in no corrosion mode, with the exception of the cases when the initial temperature of the heated air is below $20\ ^oC$. Such cases are rare because the air is sucked from the room where the steam generator is installed. The presence of high levels of sulfur in the fuel could cause corrosion but only the last lines of the heat exchanger are threatened by corrosion. That is the reason the final line set of pipes to be executed by thick-walled tubes $\phi 35/5\ mm$.

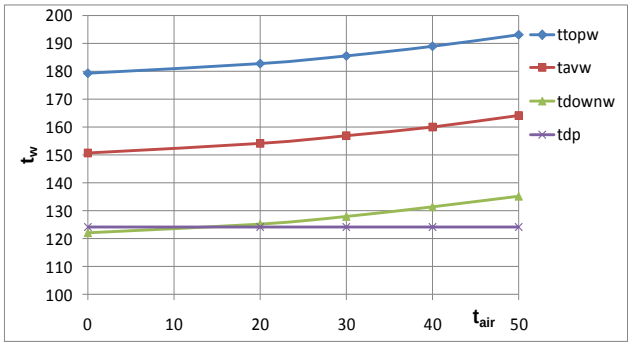

Figure 2. *Relation of the pipe wall temperature of the pipe (t_w) and inlet air temperature (t_{air})*

Table 3 shows the surface temperatures of the heat pipes as a function of temperature of the incoming air into the VP-TT when boiler production is $16\ t/h$ and temperature of the exhaust gases $160\ ^oC$.

IV. Conclusions

The performed calculation in the current work resulting in following:

- The air heater with heat pipes outperforms all conventional tubular air heaters regarding corrosion safety in all modes of operation;
- When the boiler operates at non full load regimes, the entire pipe air heater is in the danger corrosion zone and the air heater with heat tubes - partially in the lines close to the exit. This is the reason, the whole set of heat pipes AH-HP to be executed by tick-walled tubes $\phi 35/4\ mm$, ensuring high reliability of operation. The majority of the set of heat pipes of the AH-HP in most of the time operates in corrosive safety conditions;
- The designed air heater with heat pipes ensured high level of heat utilization and for the current steam generator Viessmann vitomax-200 type the utilizing power at nominal mode reaching up to 335kW providing heating of the supplied air from 20 to $100\ ^oC$, when the flue gases are cooled from 220 to $160\ ^oC$.

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INFLUENCE OF WATER ON DOUGH RHEOLOGY AND BREAD QUALITY

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Abstract: *The water content is a very important parameter of the dough. The consistency of dough depends on the amount of free water in the dough, which facilitates the enzymes mobility, reported to the substrate and therefore the intensity of biochemical processes. Too little water will lead to a too high viscosity and this will cause difficulty during dividing, handling and molding. Too much added water will lead to dough with a low viscosity and it will stick to processing equipment. The present paper aimed is studying the influence of water on the rheological characteristics of bread dough and quality of bread was studied.*

Keywords: bread dough, water content, consistency

I. Introduction

Water absorption in baked products can be defined as the amount of water addition required to produce a dough optimum for processing. Moisture levels in bread formulations influence the rheological properties of the bread dough as well the textural properties of the baked bread. It is thus important to produce a dough whit an optimum water level. Too little water will lead to a too high viscosity (the dough will be stiff) and this will cause difficulty during dividing, handling and molding. Too much added water will lead to dough with a low viscosity (the dough will be too soft and may not retain its shape) and it will stick to processing equipment. Dough water levels are also dependent on bread variety, bread making process and processing methods.

In the baked bread, moisture content contributes towards the textural properties. Perception of freshness is generally linked to the moisture content of the crumb, although bread stored under the proper conditions for several days will have the same moisture content as that of freshly baked bread. [4]

Water is regarded as one of the critical factors that contribute to bread staling. Another major factor is starch. The overall staling process can be divided into two sub processes: firming of the crumb caused by moisture transfer and intrinsic firming of the cell wall material, which is due to starch retrogradation. Bread with lower moisture content does stale faster than that with higher moisture content.

The water levels are critical when referring to bread spoilage. Too little water would accelerate staling and too much water would encourage spoilage by moulds.

If exist an excess of water, the dough will have small consistency and bread obtained will be flat.

II. Influence of water content on rheological dough properties

Rheological properties of the dough plays an important role in the production process, in which the dough is suppose to action of forces which causes stress and strain.

Water is an essential component of the dough, primarily because when mixed with flour result a mixture which mechanical behavior enables the desired formation on bread making process, and secondly because after the baking there is more or less water in bread, water which play an important role in determining the texture.

Absorbed water from flour in mixing process is in the form of water related, integral part on the structure of dough, and partly as free water, responsible for the fluidity of the dough.

Related water represent 30-35% on the total amount of water in dough, the remaining of 60-65% being under the form of free water. [1]

Largest amount of water from bread dough is related to gluten and starch. The formation of dough, the gluten must be hydrated. Optimal time for mixing grow at small dough humidity, like we can see on table 1.

Grow of time for dough develop it is bigger when the flour used are much stronger. [2] Rheological properties of the dough, elasticity and viscosity, increase up to certain values of water content, corresponding of maximum swelling of the proteins, then their value decreases. Optimum consistency is obtained when the dough contains enough water to swelling flour components. An insufficient amount of water on the dough not achieved optimal swelling of gluten proteins; dough is obtained with reduced elasticity, and the final breads will have volume and porosity underdeveloped.

Table 1. *Influence of water content on time of dough develop [3]*

Water, %	**Time for dough develop,** s
36,52	260
38,52	300
40,52	350
42,52	415

Influence of dough humidity on viscosity and storage module are presented in table 2.

Table 2. *Influence of dough humidity on rheological characteristics of dough [3]*

Dough Humidity, %	**Rheological parameters of dough**	
	Viscosity η **,** $[Pa \cdot s^{-1}]$	**Storage module [G],** Pa
36,52	$1{,}9\ 10^6$	1675
38,52	$1{,}6\ 10^6$	1640
40,52	$1{,}2\ 10^6$	1602
42,52	$1{,}2\ 10^6$	612

III. Materials and method

Materials

Three commercial wheat flour samples (FA_1, FA_2 and FA_3) of varying quality characteristics were procured from the local market, and used in the studies.

Methods

Flour analysis: moisture, ash, protein content, gluten and acidity characteristics were determined, characteristic shown in table 3.

Bread formula and ingredients

Bread dough was prepared using direct method preparation. It was preparing dough's with different consistency: dough with normal consistency, dough with soft consistency (+10% water from hydration capacity) and dough with strong consistency (-10% water from hydration capacity).

Preparation of the dough

For preparation of bread dough was used flour, water, yeast and salt. The ingredient was mixing using the Spiral Mixer Silver 50, for 8 minutes at 90 rpm, and next for 4 minutes at 180 rpm. After resting, the dough was baked at 200°C for 40 minutes.

Table 3. *Characteristics of flour used*

Characteristic	**FA_1**	**FA_2**	**FA_3**
Moisture, %	14,03	14,00	13,98
Ash, %	0,58	0,61	0,49
Proteins content, %	13,29	13,46	13,12
Gluten, %	29,3	29,9	29,5
Acidity, %	2,4	2,5	2,4

Measurement of influence of consistency on bread

After baking we measured the volume, porosity, acidity of the bread, and were also carried out a sensorial analysis of bread obtained. The results are shown in table 4.

Figure 1. *Dough with strong Consistency*

Figure 2. *Bread from dough with Normal Consistency*

Table 4. *Characteristics of bread obtain*

Sample	Characteristics Measured	Dough with Normal Consistency	Dough with Soft Consistency (+10% water from hydration capacity)	Dough with Strong Consistency (-10% water from hydration capacity)
FA_1	**Volume**	305	320	290
	Porosity, %	79	82	73
	Acidity, degrees	2,7	2,9	2,6
	Sensorial Analyses	Taste and smell specific; uniform porosity	Taste and smell specific; uniform porosity	Taste and smell specific; brittle crumb
FA_2	**Volume**	290	275	263
	Porosity, %	73	75	70
	Acidity, degrees	2,5	2,7	2,4
	Sensorial Analyses	Taste and smell specific; uniform porosity	Taste and smell specific; uneven porosity	Taste and smell specific; brittle crumb
FA_3	**Volume**	320	308	304
	Porosity, %	76	79	75
	Acidity, degrees	2,5	2,6	2,3
	Sensorial Analyses	Taste and smell specific; uniform porosity	Taste and smell specific; uneven porosity	Taste and smell specific; brittle crumb

IV. Results and discussion

The results are presented in graphic form.

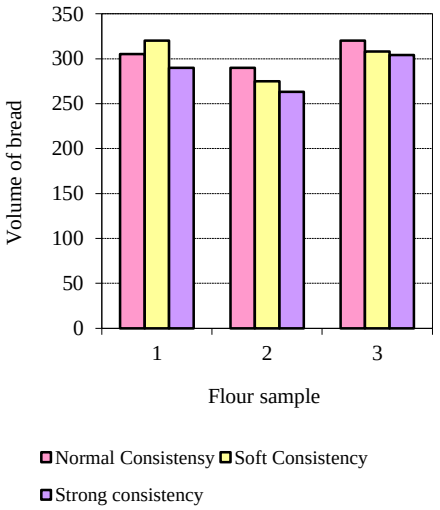

Figure 3. *Influence of water added in dough on bread volume*

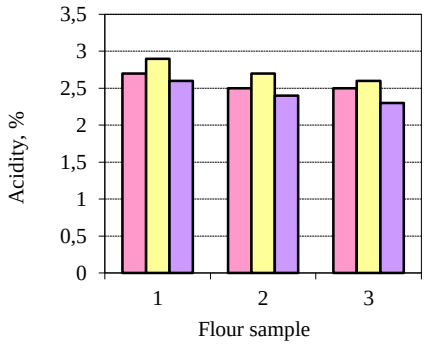

Figure 4. *Influence of water added in dough on bread acidity*

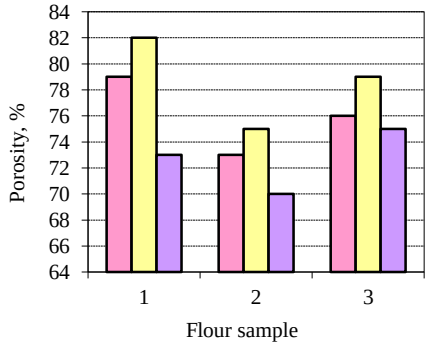

Figure 5. *Influence of water added in dough on bread porosity*

V. Conclusion

The result obtain under modification of dough consistency realized with added water with 10% to much than hydration capacity of flour, respectively with reducing with 10% of water content than hydration capacity, we can observe that the quantity of added water can influence the quality of the bread.

It has observed that for the bread obtain from flour FA_1 and FA_2 with grow of quantity of water comparatively with hydration capacity have a positive influence on volume and porosity of the bread. Insufficient amount of water on the dough has a negative influence on the bread.

The organoleptic points of view the bread obtained from all 3 types of flour with added water have suffered the same modification; the taste and smell were specific to the bread.

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SOME PROSPECTS OF PULSED ELECTRIC FIELD TREATMENT IN FOOD PROCESSING

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Abstract: *High intensity pulsed electric field (PEF) preservation technology is an innovative and perspective method, based on the capacity of such fields to break cell membranes, causing lysis of microbial cells. PEF technology can be regarded as a possible alternative to traditional thermal pasteurization processes with the benefits of minimizing deterioration in taste and nutritional properties. On the other hand law intensity PEF can increase the yield of production of some commercially interesting and high value metabolites and useful enzymes (milk-clotting, laccases, tyrozinases, etc.)*

Key Words: pulsed electric field, microorganisms, pasteurization, yield of methabolites.

I. Introduction

Electromagnetic fields in all frequency ranges influence living organisms and biomolecules in one way or another. The influence of the energy fields of different nature on microbial cells is attracting growing interest. The variety of effects of the influence of external fields on the microorganisms are described in numerous sources, shows the relevance of the development of this studies.

Effect of various external fields on the organisms is an environmental norm. During the evolution process mechanisms of adaptation to such impacts have been developed. However, scientific and technical progress on the one hand leads to new challenges, while on the other to the emergence of new opportunities to influence the growth and development of organisms through artificial fields.

Due to developments in food technology, technical microbiology and biotechnology the influence of artificial fields on microorganisms is attracting growing attention.

Activity of microorganisms is a major cause of food spoilage. Spoilage can cause not only huge economic losses, but also can threaten people's lives and health. The traditional heat sterilization or pasteurization usually results in varying degrees of loss of some heat-sensitive materials, nutritional content of food products and certain characteristics.

First attempts to study the influence of electricity for food preservation processes were taken since almost from the time when electricity became commercially available. It was first applied for the pasteurization of milk using a process known as the Electro-Pure method [1-3]. However, this method was practically a thermal process as the milk was heated up by ohmic resistance [4]. Only those researchers who had applied high voltages have been reported the ability of the process to kill the bacteria “below their thermal death point” [5,6].

In early 1980s several papers discussing the sensitivity of various bacteria to PEF were published [7-9]. PEF processing involves the influence of pulses of high voltage to foods placed between two electrodes.

Since that time PEF treatment, as a nonthermal method of processing of foodstuff, has been of increasing interest. This method shows the potential to provide consumers with microbiologically safe and quality foods. In terms of food quality, PEF technology is considered superior to traditional heat treatment of foods, as it excludes or significantly reduces the unwanted changes of the sensory physical and nutritional properties of foods [10]. Application of high-voltage electric fields at a certain level for a very short time by PEF not only inhibits the growth of pathogenic and spoilage microorganisms but also results in the preservation of flavor, aroma, nutrients, and color of foods [11].

A typical PEF system consists of the following components: a high-voltage power supply, a pulse generator, a number of energy storage capacitors, treatment chambers (either static or continuous) that house the electrodes, a pump to pass the liquid food through the treatment chambers (if the system is continuous), cooling and heating baths, measurement devices (voltage, current and temperature), and a central process unit to control

operations. High field intensities are achieved through storing a large amount of energy from a DC power supply in a capacitor bank, in series with a charging resistor which is then discharged in the form of high voltage pulses [12].

Application of PEF for inactivation of vegetative cells of microorganisms has been widely studied during last decades. Successful results were obtained in processing of various liquid products, such as milk, juices, liquid eggs, and pea soup [13-21].

In addition the implementation of PEF appears to be very promising in the recovery and production of various commercially interesting and high value metabolites (natural pigments, sugar) from food [22-25], the improvement of the yield of fruit an vegetable juices in solid-liquid extraction [26-30], intensifying diffusion [31-32] and acceleration of mass transport in drying processes [33-38].

The mechanism of inactivation of microorganisms by PEF is not yet fully identified and knowledge on microbial inactivation mechanism is very important to design and develop more efficient PEF technologies and equipment for efficient inactivation of microbes in foods [39-42]. According to several publications this inactivation of microorganisms is mainly related to the electromechanical instability and changes in the cell membranes [43-44].

When high voltage pulsed electric field is applied to the product placed between two electrodes the pores of the cell membranes of microbes are irreversibly enlarged, which kills the cells and releases their contents into the environment. Cell membranes are one of the vital parts of the cells that enclose and protect the constituents of a cell. This membrane physically separates the contents of the cell from the outside environment and gave the shape to the cell. Cell membranes have pores, throw which the nutrients flow into, and metabolic wastes flow out of the cell. If these pores become wider and larger, the cytoplasm can leak into the medium, thus killing the cell. It is impotent to note, that PEF method is efficient only against vegetative cells, spores are more resistant to this method. The temperature rise in PEF pasteurization system is less than 30°C and therefore the treated material does not achieve pasteurization temperature sufficient to kill microbial cells by heat alone. The voltage applied can be between 15000 to 30000 volts, where the lower voltage can kill plant cells while the higher voltage brings about the death of bacterial and fungal cells.

Several factors are affecting microbial inactivation during PEF treatment: microorganism type and growth stage, environmental and processing parameters.

The Gram-positive bacteria are more resistant to PEF treatment than Gram-negative ones [9]. Yeasts are more sensitive to the influence of electric fields than Gram-positive bacteria due to their larger size, but they seems to be more resistant than Gram-negative bacteria [16, 45]. Spores are the most difficult ones to inactivate by PEF treatment and even combination of heat around 60°C with 75 pulses of 60 kV cm^{-1} could not inactivate B. cereus spores [46, 47]. Another factor to be considered in PEF treatment is the growth stage of microorganisms. Cells are more sensitive to stress during exponential phase of growth than at the lag phase or stationary phase. Microbial growth in exponential phase is characterized by a high number of cells undergoing division, during which the cell membrane is more sensitive to the influence of applied electric fields [9, 48].

The field intensity, pulse wave shape and polarity, total specific pulsing energy input and treatment time are among the process parameters, affecting efficiency of PEF treatment (49).

Critical external field intensity required for microbial inactivation is highly dependent on the cell size as well as the field orientation [50]. Inactivation of smaller cells requires higher intensity of the field. The orientation of the rod-shaped cells along or across the electric field lines also influences the required field intensity [51].

Pulse wave shape is also affecting the microbial inactivation. Square pulse waveforms are more lethal and more energy efficient than exponential decaying pulses. Oscillatory decay pulses are the least efficient, because they prevent the cell from being continuously exposed to a high intensity electric field for an extended period of time, thus preventing the cell membrane from irreversible damage over a large area [52].

The energy input and treatment time are linearly correlated and increasing of each of them can result in increased microbial inactivation [21, 53].

Environmental factors, such as treatment temperature, conductivity and ionic strength, pH of the medium also have significant impact on PEF treatment efficiency.

In several studies it was shown, that PEF treatments at moderate temperatures (50 to 60°C) exhibit additive effects on the inactivation of microorganisms [54- 57].

The combined effects of low pH and organic acids can enhance the inactivation of PEF technology on spoilage microorganisms. However, as most of organic acids are preservatives themself, it is possible that the inactivation levels achieved were also due to the chemical properties of the acids.

The effect of pH on the sensitivity of microorganisms to PEF treatment is likely dependent on the target microflora [58].

In the media with high electrical conductivities, during PEF treatment electric fields with smaller peaks are generated which do not cause the required killing effects [59]. An increase in ionic strength of a liquid leads to increased conductivity, thus resulting in a decreased microbial inactivation level. So, the inactivation level of microorganisms increases with decreasing of the conductivity of the medium [60].

In our recent studies it was demonstrated, that law intensity PEF (up to 1000 volts) applied during the cultivation of basidial fungi can increase the yield of commercially valuable enzymes, such as milk-clotting enzymes, laccases, tyrozynases.

Although PEF preserves the nutritional components of the food, and allows to increase the yield of production of some useful metabolites [61], its several areas need further research before the technology is applied commercially. Effects of PEF on the chemical and nutritional aspects of foods still must be better understood before it could be used in food processing. Nevertheless, there is no dought, that high voltage pulsed electric fields, may definitely be used for food preservation and pasteurization, to maintain food quality to the maximum level.

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IR-DRYING OF NON-TRADITIONAL PLANT RAW MATERIAL

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Abstract. *Results of the research of investigating IR-drying of non-traditional plant raw material are presented and rational parameters as well as factors influencing quality of the received products are determined.*

Keywords. IR-drying; factors influencing the process

Research objectives defined

Nowadays the main purpose of food industry is to raise biological value of foodstuff, which is caused by environmental degradation in Ukraine and other European countries. One of the ways to increase foodstuff quality and improve the structure of population alimentation is to introduce new non-traditional types of plant raw materials containing a complex of proteins, lipids, minerals, vitamins, which possess high nutritive, medicinal-prophylactic properties and palatability.

Rowan, chokeberry, whitethorn, viburnum, sea buckthorn and others are among the most perspective types of non-traditional plant raw materials. They cannot be used all year round because seasonality of agricultural production of vegetative and berrylike material is the characteristic feature. Water extraction from wild-growing raw materials by means of drying to the humidity level 4….4,5% will give an opportunity to maintain it in customary conditions for a long time.

Drying technology and its parameters are of great importance for the obtaining of dried fruit and berry products in which all valuable components of initial stock and its main properties are maintained to the maximum.

IR-drying as technological process is based on the fact that infra-red radiation of the determined wave-length is actively absorbed by the water contained in the product but not absorbed by the tissue of a dryable product. Dehydration is possible at low temperatures (40…600C) that allows maintain vitamins, biologically active substances, natural color, flavor and aroma of the dryable products. IR-drying allows maintain vitamins and other biologically active substances in a dry product at the level 80…90% of the initial stock. During short moistening (10…20 min) the product renews its natural organoleptic, physical and chemical properties and can be used fresh or culinary treated. In comparison with traditional drying, non-traditional fruit and berry raw material treated by infra-red light maintains flavor close to the fresh one. Besides, powders treated by infra-red light possess antiphlogistic and antioxidant properties. A dried product is not crucial to storage conditions and persistent to the development of microflora.

Literature review

Drying may be chosen as one of the perspective methods for the storage of foodstuff with a large amount of BAS in order to increase their biological value. Theoretical and experimental investigations proved that application of IR-radiation can considerably increase quality of ready products in the technology of drying fruit and berry raw materials [2].

Nevertheless both properties of the received dried products and peculiarities of the equipment for drying foodstuff by means of IR-radiation and technological processes based on this principle deserve much attention. IR-drying of wet products allows using 100% of energy linked up to a dry product. Water molecules in the product absorb infrared rays and warm up, i.e. unlike in other types of drying, the energy is brought directly to the water of a product, which results in a higher coefficient of efficiency. It means that there is no necessity to raise the temperature of the product, which is to be dried, the process of drying may occur at temperature 40…600C. Such drying has two preferences: first, the product is maximally saved at such temperatures – the cells do not break, vitamins are not killed, sugar is not caramelized; secondly, low temperatures do not heat drying machinery, i.e. the warmth is not lost through the walls or ventilation. At the same time IR-radiation at temperatures 40…600C allows to kill microflora on the surface of the product making it sterile. [2].

Besides, drying machinery is universal, permits process any fruit and berry, and receives fast renewing dry products. Drying equipment has a number of advantages:

- the lowest power intensity per 1 kg of evaporated moisture;
- less than 1 kWh/kg (two times less than any other drying machine);
- drying products at low temperature 50…600C;
- high speed drying – 30…200 min.

Research objectives defined

The objective of the research is to investigate and analyze experimental data concerning IR-drying of nontraditional fruit and berry raw material and specify main significant factors influencing the result of drying process.

Research methodology

Process scheme of experimental works contains the following operations: dishwashing, determination of starting content of moisture in a product; weighing a product; IR-drying under cylindrical reflector on different heights of a tray; different distances between the radiator and reflector; different thickness of raw material layer and determination of final moisture content in a product.

Use of infrared treatment of fruit and berry raw material permits intensify drying process and get semi-finished products with significant vitaminous properties. At the same time the influence of various factors on the process was experimentally checked, the data with the determination of main mathematical model of the process of drying nontraditional fruit and berry raw material with the determination of the importance of factors and adequacy of mathematical description were analyzed.

Experimental installation was used for the experiments (fig. 1). It consists of a process chamber 1, cylindrical reflector 2, trays' guide ways 3, branch pipe for condensate extraction 4, IR-reflector 5, tray for the product 6, thermal couple 7, temperature controller "TRM1" 8 and assembly pin 9.

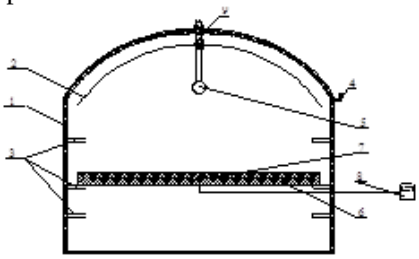

Figure1. *Experimental installation for IR-drying of wild-growing fruit and berry raw material*

Fruit and berry raw material, namely chokeberry was chosen for the analysis of drying as a process. Determination of starting humidity was the first step of the experiment.

Then the samples were weighed and dried at temperature 60…650C under quartz reflector with power capacity 1 kW. After that the samples were weighed every 5 minutes. The distance from the reflector's top to the tray with a product equaled 175, 225 and 275 mm, thickness of a product's layer was 7,17 and 24 mm.

Residual humidity of the product stabilizing during the drying process was chosen as response function Y. Statistic treatment of experimental data resulted in linear regression equation (1), which imposes a constraint between the factors of the experiment and response.

$$Y = 45{,}0866 + 0{,}0441 \cdot X1 - 0{,}0347 \cdot X2 - 0{,}2825 \cdot X3 + 8{,}3972 \cdot X4 \quad (1)$$

The hypothesis is reliable, which is proved by Fisher's ratio test (signification F< 0,5 %). Correlation and determination indexes equal correspondingly 95 % and 90 %. So, final humidity directly and substantially depends on cumulative influence of the chosen factors [3].

According to statistical importance of the experiment the most influential factors are drying time, distance of the product to the radiator and thickness of raw material layer. Statistical insignificance of the other factor by no means is the reason to exclude it from the plan because it will result in the change of regression equation. Statistical importance of scholastic mistake indicates that all the factors influencing the response as well as plan of the experiment were determined, and do not require broadening of the factors.

Conclusion

Analysis of the experiment demonstrated which of the parameters and assumptions influence quality and indexes of work. The distance between the reflector and the product, the distance between the radiator and reflector, thickness of the product's layer on the tray and duration of drying are the main factors significantly influencing the technological process.

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PERSPECTIVE DIRECTION OF COMPLEX IMPROVEMENT OF RUSK WARES

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Abstract: *The necessity of complex perfection of process of production of rusk warest* comes up *from the traditional method of production,* wide usage *of hand labour and bulky equipment. The process of mixing of dough, treatment of dough purveyances, baking and drying is investigational by us. It is set that the intensive mixing of dough allows to shorten duration of his fermentation, the using of dynamic method of* loosen of dough purveyances *and combined process of baking baking-drying . On the basis of the conducted researches the machine-instrumental chart of the complex stream-mechanized line of production of rusk wares is offered*

Keywords: mixing of dough, treatment of dough purveyances, baking, drying, intensive, complex stream-mechanized line

I. Introduction.

Lately the expansion of demand and variety of rusk products, especially it touches the wares of small diameter. However for their production traditional technology and equipment are used. Continuous forming of dough cord is interrupted for a portion stowing of flags on sheets for del standing on the cradles of proofers. The duration standing for the rusk sleepers of small diameter in a few times exceeds duration of baking of this sort of rusks, that means that providing of the productivity of stove an area proofers must be in a few times more area of hearth del, and taking into account the small geometrical sizes of wares the volume proofers is used very uneffective.

The baked sleepers are maintained during 4 - 8 hours before cutting before cutting.

To the present times the question of cutting of rusk sleepers of small diameter in a stream is not solved. After cooling and standing rusks are cutting and that cause rising of hardness as a result of staleing. In addition the repeated heating of rusk hunks to the temperature of evaporation after cooling of rusk flags before cutting cause the additional charges of heat.

The disadvantage of crackers in the traditional way is the need of large industrial areas and a large number of production personnel.

These problems need to be solved. It can be done by a comprehensive study of all processes of production of rusk products, and apply research results to improve the instrumentation of the whole production cycle, from dough preparation till packaging of rusk products.

II. The research of complex improvement of rusk products

The practice of baking companies shows widespread adoption of intensive technologies of preparing dough in kneading machines with intense action. Without deterioration of the finished products without dough way to prepare the dough can be successfully used for rusks products.

Using our proposed method of production and distribution with loosening in dynamic conditions will ensure continuity of process handling dough purveyances.

The production of thin products baking and drying processes are mixed in a baking chamber and run consistently. Investigation of drying and baking dough purveyances of various shapes and sizes combined in a single baking chamber is needed to determine the size and conditions of heat treatment of products in which it is expedient to combine baking and drying processes for more efficient energy use and production areas

2.1.Research of the process of dough preparation and processing dough purveyances

We investigated, the proposed experimental setup for intensive kneading, which ensured that all three stages of kneading dough. The working body consists of three parts: the tape, screw with variable step. Tape provides intensive mixing of components in the initial stage, actually mixing may occur at rest without significant expenditure of energy, so screw design covers the transportation of dough to the next stage. Plasticization are provided by intensive influence on dough screw with variable step

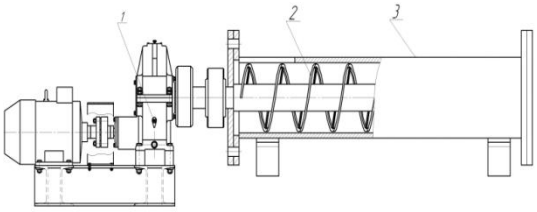

Fig.1. *There is a chart of experimental fluidizer research of intensity of treatment of dough. 1 is an occasion; 2 is a working organ (scruw); 3 is a corps;*

The main indicator of the structural and mechanical properties the dough is the effective viscosity. We have conducted the research to determine changes in viscosity of the dough, depending on the specific work flow per knead.

Analysis of experimental data (Fig. 2.) showed that the viscosity of the dough is decreasing while the intensity of machining is increasing due to the weakening of connections between the particles of the dough and the forces of viscosity is overcoming by increasing of the kinetic energy of the molecules.

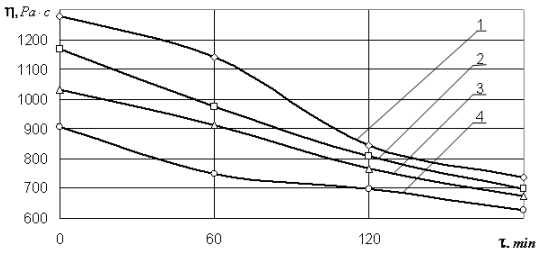

Fig. 2. *Dependence of the effective viscosity of the dough from the time of fermentation when the specific work is: 1 - 7.5; 2 - 15; 3 - 22.5 ; 4 - 30 J / g.*

As a consequence from the experimental data, reducing of viscosity occurs also during the time of fermentation of the dough and especially intensively during the first hours of fermentation. The value of viscosity after 1 hour of fermentation with the consumption of 30 J / g specific work per batch is the same that is during 3 hours of fermentation with consumption of 7.5 J / g specific work per batch. Therefore, changes in the structural and mechanical properties of dough which occurs during the fermentation process due to repeated stretching during the formation of gas bubbles can be achieved by intensive mechanical treatment of the dough during kneading.

Application of the intensive mechanical treatment during the dough mixing process allows to reduces the process of fermentation of the dough and to distribute yeast cells more uniformly throughout the whole volume of the dough which , which promotes the formation of more centers of gassing and obtain a uniform fine-pored structure finished products.

The research process handling dough pieces in dynamic conditions was carried out using the experimental set forth in earlier studies [3], which allows a wide range of both kinetic and dynamic characteristics

Based on the research process and kneading dough processing blanks we proposed structure pf Mixing-Fermentation-forming unit, which sequentially processes of intensive kneading, fermentation and formation extruding loosened dough cords in the tunnel furnace.

2.2.Researching of combined baking and drying process.

Baking and drying of rusk products took place in the baking chamber laboratory settings. Bakery camera has a top and bottom of the heating surface, wich corresponding the principle in most industrial tunnel ovens, for which both are offered this method of construction rusk products.

Experimental setup for baking and drying of rusk products (Fig. 3).

A stove has a hot automatic supply of heat to separate the upper and lower surfaces of the heating.

Temperature measurement in layers of dough cords are thermocouples, the potential of which goes to the analog module ICP CON I-7018 11 with conversion module ICP CON I-7520 12 signal is converted and transmitted to the computer.

To determine the intensity of external heat mass transfer on the results of experimental studies dough pieces of cylindrical shape, length 0.25 m and different average diameter, which varied from 0.017 to 0.06 m length was studied combined process of baking, drying, depending on the size determinant in different temperatures bakery camera.

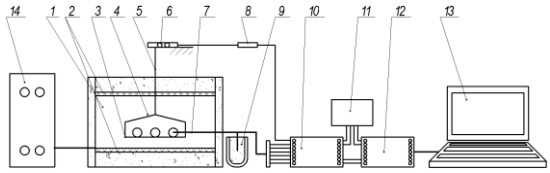

Fig.3. *There is a chart of experimental fluidizer baking and drying of rusk wares.*
1 is a baking chamber, 2 - overhead and lower warming surfaces, 3 - bakestone, 4 is a pendant, 5 is a barbell, 6 is a gravimetric strain gauge sensor, 7 is a block of thermocouples, 8 is a strengthener of signal, 9 - Dewar flask, 10 is the analog module, 11 is the power module, 12 is the module of transformation, 13 is computer, 14 is a management corymb.

Analysis of experimental data shows that the baking time is 20-25% of the total duration of the process depending on the size of the determinant.
To determine the limits of geometrical dimensions in which it is expedient to combine the processes of baking and drying rusk plates in a baking chamber perform a comparative analysis of processes of baking and drying tusing the traditional method and proposed one. This identified the duration of drying rusk slabs and slices with equal limits defining dimensions. Rusk plates are shaped with the help of extrusion with determining size from 0.01 to 0.028 m length of drying rusk plates during the baking, drying, defined as the difference between the total duration of the process and duration of baking.

For slices of slab shaped extrusion with an average diameter 0,065-0,07 m and bake until cooked, cooled for 1 hour and cuting in slices of varying thickness. Then cut slices rusks dried and identified the duration of the process.

From the obtained comparative curves (fig. 4) duration of dry slices and bundles at different temperatures shows that for rusk panels this dependence is power-law character. With the increase of the equivalent amount the drying time increases for both samples, but the drying cords increases more rapidly compared with slices that have a linear dependence on the length of the baking equivalent size.

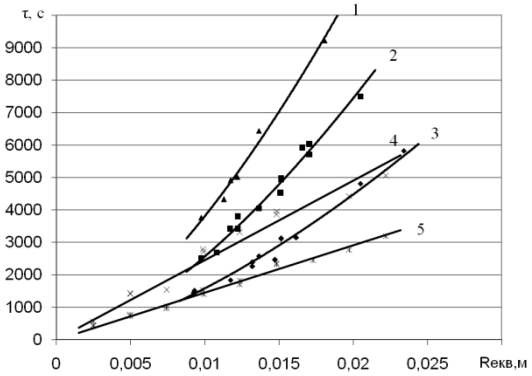

Fig.4. *Duration of drying of rusk flags and hunks depending on a qualificatory size: 1,2,3 - rusk flags at the temperature of baking chamber according to 120, 150, 190 ºC; 4,5 - hunks at the temperature of baking chamber according to 150, 190 ºC.*

Dependences of the duration of drying of rusks slabs and slices of equivalent size and ambient temperature Baking chamber in the range studied the values described by equations (1) and (2) respectively:

$$\tau_{суш}^{пл} = (-34264 \cdot t + 8.1 \cdot 10^6) \cdot R_{екв}^{1,5} \quad (1)$$

$$\tau_{суш}^{ск} = (-2492 \cdot t + 0.619 \cdot 10^6) \cdot R_{екв} \quad (2)$$

For the interval of values of equivalent size Rekv 0,01-0,013 = m characterized by a critical point at which the observed cross section curves of the length of the drying process. Before reaching the crucial importance of the size of the drying process suharnyh plates is faster compared to the drying slices. We believe this phenomenon is associated with a stage heating to a temperature of chilled slices of evaporation that takes place at the beginning of the drying process. With increasing size of determining the impact of this factor is reduced and consequently the drying suharnyh plates begins to exceed the drying slices

2.3. Create a stream-mechanized production line suharnyh products

On the basis of the studies we proposed machine-instrumental chart of production of rusk wares (fig.5), establishment of, Mixing-Fermentation-forming unit in that the intensive premix of dough, fermentation and forming of

loosening of dough plaits passes on under stoves, is foreseen in that. In a stove continuous rusk flags are baked and dried out, cut on separate slices, if necessary dry and packed.

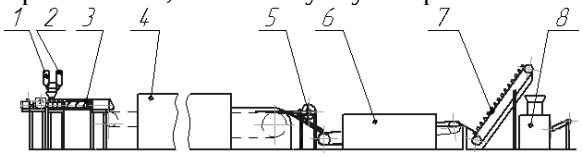

Fig.5 *Threaded-mechanized production line suharnyh products: 1.2 - dispensers, 3 - Mixing, Fermentation molding unit 4 - oven, 5 - Cutting Machine, 6 - camera dosushuvannya 7 - transporter, 8 - packing machine*

III. Conclusions

Thus, the conducted researches allowed to carry out complex perfection of process of production of rusks :

- use of intensive premix of dough,
- loosen dough in dynamic terms,
- forming extruding of gasfilled dough;
- creation of mixing-fermentation-forming unit;
- combination of operations of baking and drying is in one stove unit.

Practical introduction of results of researches is creation of the stream-mechanized line for the production of rusks.

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INFLUENCE OF ACIDIFICATION OF THE QUALITATIVE CHARACTERISTICS OF CARAMELIZED CANDIES

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Abstract: *The impact of organic acids commonly used as additives in the production of caramelized candies has been studied. The influence of acidification process of caramelized mass with mixture of citric and malic acid and only with citric acid on the quality characteristics of the finished product has been determined. It is estimated that acidification increases the reducing substances and hygroscopic properties of caramelized candies.*

Keywords: caramelized candies, citric acid, moisture kinetics, reducing substances

I. Introduction

Caramelized candies, also known under the name of hard candies occupy a significant share of the confectionery market. They are mainly preferred because of their great diversity in terms of taste, rich aromatic qualities and functional properties. Caramelized candies are characterized with refreshing and deodorizing properties, and capability to stimulate the salivary glands in the mouth. Due to their relatively low prices, hard candies are known as "democracy sweetness".

Quality characteristics of hard candy are largely determined by the properties of half-finished product - caramelized mass, as well as of its additional treatment. It includes technological treatments like coloring, flavoring and acidification.

Acidification is used widely in the production of caramelized fruit flavored candies (lemon, orange, strawberry, raspberry, apple, etc.). This involves the use of organic acids recognized as permitted food additives [1]. In this case, the acids used for acidification must fulfill certain requirements - to have a low inversion capacity, to be relatively thermo stable, not to be highly volatile and to be highly soluble in water. In the past the acidification had been performed more commonly with Tartaric acid (E334), but nowadays are mainly used: monohydrate citric acid (E330), malic acid (E296) and lactic acid (E270) under certain conditions. The main reason for use of monohydrated citric acid is its relatively low melting point temperature (70°C). This allows its homogeneous and uniform distribution throughout the volume of caramelized mass [2,7]. Flavor profiles of citric and tartaric acids are close and give a sharp, but not prolonged sensation of sour taste. Malic acid is characterized with less pronounced initial sour taste, but the taste remains for a long time [6].

According to Marshalkin [3], the process of acidi fication due to catalytic action of acids influence the inversion of sucrose and the content of reducing substances in the half-finished product (caramelized mass) increases. This will undoubtedly affect the indicators that define the properties of the final product - caramelized candies.

The aim of this study was to determine the influence of the acidification process on the qualitative characteristics of caramelized candies.

II. Materials and methods

For analyzes were used caramelized candies produced in industrial conditions in the technology adopted in the candy factory "ALPI", Asenovgrad. Acidification of caramelized mass before molding candies has been conducted giving two options. In the first case (Variant 1) caramelized mass is acidified with citric acid monohydrate. In the second option (Variant 2) is used the same amount acid agent: a mixture of monohydrated citric and malic acids, respectively, in terms 0,7 : 0,3. In both cases is used half-finished product - caramelized mass - 30 kg. To determine the effect of acidification process the main physicochemical characteristics of caramelized mass before its acidification and the main physicochemical characteristics of moldy candies after coloration, flavoring and acidification of half-finished product (caramelized mass) have been determined. The values of these characteristics - total sugar content, reducing substances, sucrose, and active titratable acidity have been determined by methods in [2].

Determination of hygroscopic properties of the samples has been carried out by analyzing the kinetics of moisture at 25 °C and a relative humidity RH = 75,3% using the methodology for determining the equilibrium moisture content as it is described in

[2]. To maintain a constant RH saturated solution of NaCl has been used [5].

III. Results and discussion

The results of the analyses of physicochemical characteristics of caramelized mass before its acidification and the main physicochemical characteristics of moldy candies after coloration, flavoring and acidification of half-finished product (caramelized mass) are listed in Table 1. and Table 2.

Data in the tables show that in both samples the values of caramelized mass and the resulting candies are closed. The data also shows that the content of total sugars after acidification practically does not change. The amount of reducing substances is increased in absolute value of 1.5% for both samples. In the first case the sucrose content decrease with 1.1 % and for second case with 2.1%, this may be explained by a partial inversion of its acids. Understandable after acidification, titratable acidity increased and the active acidity (pH) decreased in relatively large range.

The properties of caramelized candies to absorb moisture from the air, the amount of adsorbed moisture and the rate of adsorption is determined by many factors. One of these factors is their chemical composition. It affects the solubility of substances in the adsorbed water [3,4]. In this regard, a comparative analysis to determine the impact of acidification on the hygroscopic properties of caramelized candy by moisture kinetics has been conducted (Fig. 1). Used samples are crushed caramelized candy mass with size fraction from 1.5 to 2 mm.

Diagram in Figure 1. shows that acidification affects the moisture sorption capacity of candy.

Table1. *Values of measured physicochemical characteristics of caramelized mass (I.1.) and caramelized candies (I.2.) acidified with citric acid.*

Physicochemical characteristics	***Case 1***	
	I.1.	***I.2.***
Humidity, %	2,8	3,1
Reduced substances, % (inv. sucrose) on dry matter	13,5	15,0
Total sugar content, % (inv. sucrose) on dry matter	80,0	80,4
Sucrose content, % on dry matter	63,2	62,1
Titratable acidity grad. on dry matter	0,7	8,7
pH	6,1	2,9

The influence of different types of acidification (by citric acid and a mixture of citric and malic acids) on the hygroscopic properties of the candies has been studied.

Table 2. *Values of measured physicochemical characteristics of caramelized mass (I.1.) and caramelized candies (I.2.) acidified with mixture of citric and malic acids.*

Physicochemical characteristics	***Case II***	
	II.1.	***II.2.***
Humidity, %	2,5	2,6
Reduced substances, % (inv. sucrose) on dry matter	13,2	14,7
Total sugar content, % (inv. sucrose) on dry matter	80,6	79,9
Sucrose content, % on dry matter	64,0	61,9
Titratable acidity grad. on dry matter	0,6	9,8
pH	6,1	2,9

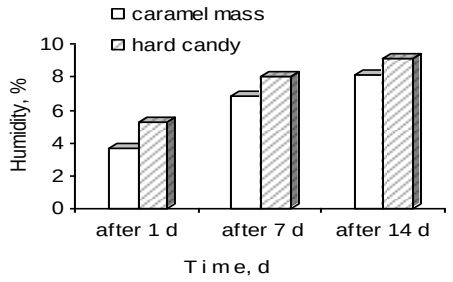

Fig.1 *Kinetics of moisture sorption of caramelized mass and caramelized candies acidified with mixture of citric and malic acids.*

Hygroscopic properties of whole caramelized candies determined by moisture kinetics are shown on Fig 2.

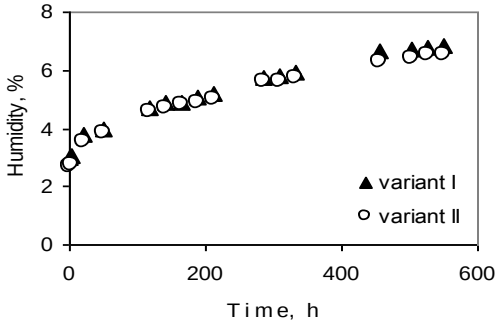

Fig.2. *Influence of type of acids on kinetics moisture sorption of hard candies*

The strong graphical correlation shows that different options of acidification virtually do not effect on the hygroscopic properties of the sweets.

The results of comparative consumer organoleptic evaluation samples show a preference for candy with a mixture of citric and malic acids. They are defined as candy with "soft", "sweet" and "persistent" sour taste.

Conclusion

The results of the research lead to the conclusion that when produced in industrial conditions caramelized candies acidification leads to an increase of reducing substances and affects their hygroscopic properties.

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ACTIVE PACKAGING WITH FOOD ADDITIVES

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Abstract: *Several studies have been conducted on active food packaging and the use of food additives in technology of their application in food production. Various functional groups of food additives such as preservatives, antioxidants, flavor enhancers and flavorings, antibiotics and essential oils, and other oxygen acceptors have been regarded as important elements of active packaging. It was found that food additives used in active packaging allow to extend the shelf-life of foods by protecting them from microbial spoilage, to improve their organoleptic qualities and keep valuable biologically active substances in products in order to satisfy the growing requirements of consumers.*

Keywords: active packaging, food additives, preservatives, antioxidants, enzymes

I. Introduction

The main task of the food industry is the production of high quality food products with fully preserved biologically active complex and very good organoleptic properties. This depends largely on the packaging technology and used materials, their ability to maintain high quality and safety of the finished products and their ability to extend the shelf-life of products. In addition, packages that get in direct contact with food must also meet safety requirements [3, 5, 7].

In the recent years the large development of equipment and technology for the production of packaging materials led to the expansion of the functions of packaging. Package is not only inert barrier between food and the environment, it is increasingly became a factor which is capable of regulating the temperature of heating food in a microwave field. With technologies of modern packaging is created optimal gas environment for each type of product inside the package and this method finds application in products manufactured in modified atmosphere gas and controllable environment [3, 8, 9]. There are various possibilities for changing food content using immobilized ferments, food additives which are implemented in the package etc. These packages are named “Active packages”.

II. Summary

The idea for active packaging technology exists for about 100 years ago but only in last two decades, this technology marks a significant progress [4, 6]. According to Dr. Werner Henlih.

Active packaging is the technology of 21st century. He points out that unlike “Smart packaging” where are used indicators for temperature measure ment or Temper Proof Systems, the term "active packaging" refers to the packaging where are used different chemical agents which induce specific properties on the package [10].

While conventional materials only act as a barrier against contamination of packaged food with them, the active packaging have many other important properties. The active packages are applied in combination with different chemical substances, which are not only permitted food additives. Some of these packages interact with food in order to prevent it from microbial spoilage and others are equipped with devices that give accurate information to the consumer about the quality of the product. For example, they may assume oxygen in the package, and thus to delay the process of spoilage. Third give information if meat is stored in a proper temperature. There are also packages that inhibit and destroy bacterial micro flora and may indicate when food starts to go spoiling [1, 10, 13, 15].

Examples of achievements in the development of active packaging using representatives from different functional groups of food additives are many.

Under development are indicators that monitor gases released into packages of frozen food. They give signals if it starts rotting in fish, meat or vegetables caused by damage of freezer [11].

There is also, an American packaging company that developed membrane that changes its permeability when temperature change and thus maintaining optimum ratio of oxygen and carbon dioxide for the proper storage of fruits and vegetables [8, 9, 12, 14].

Canadian company "Toxin Alert" even developed a package destined for sandwiches that changes colors if in the products appear microorganisms [11].

Active packaging technology involve the use of different food additives of the group of preservatives, antioxidants, flavors, perfumes etc., that migrate

from the package into food product in order to protect it, against harmful micro flora, auto-oxidation of fats and fat-containing products. The effect is extension of product shelf-life, without risk for consumer's health and improvement of food organoleptic properties.

In Russia, an original technology has been developed for the use of antimicrobial protective coating which is placed between package and food surface. This prevents hard or processed cheeses, smoked sausages, cooked sausages or deli from microbial spoilage [10].

In active packaging are used also oxygen acceptors which purify the oxygen from inside the pressurized container. The first oxygen acceptor used until nowadays is a product of Mitsubishi "Eynzheles". This product present individually wrapped iron (15/15 mm), which is oxidized into Fero-complexes. For its application is important for product to have high water activity, in order to oxidize iron. In this case the level of oxygen is 0.01% [10].

As oxygen acceptors are used photosensitive paints, ascorbic acid, ferrous carbonate, copper sulphate, etc. In dairy industry, this packaging technology can be used for wrapping cheese.

The company "AMERICAN KEN Ko" develops packaging film to remove oxygen, where is used a platinum catalyst to react hydrogen and oxygen. This technology is applied to extend shelf life of solid food for the military. The disadvantage is the high cost of platinum.

Important role in active packaging has films coating. In this technology during storage of food are released active substances which protect inside product-preservatives with antimicrobial activity against bacteria, yeasts and molds that cause degradation of foodstuffs.These preservatives are deposited as a layer on the surface of the food product and inhibit their microbial spoilage. In that reason are also used antibiotics pimaricin and natamycin (this one is approved for use as a food additive). These substances are applied for extending shelf-life of ripe cheese and some sausages. In this type of packaging there is no need for importing preservatives in food [2, 7].

For extending the shelf-life of frozen foods a significat role play water reducing film coating substances. Company "SHOVODENKO & Co" develops this film coating and it presents "sandwich" of two layers of polyvinyl alcohol, stuck on the end, between which there is propylene glycol. This additionally contributes to a lack of conditions for the development of harmful microflora [10].

Scientists from the University of Zaragoza found that in the fight against fungus growth on packaged bread better alternative than treatment with ultraviolet light or sterile packaging is a new paper packaging bearing a layer containing cinnamon oil. This new package is more reliable, safe for consumers and it is not harmful for the environment. This excellent effect is due to cinnamon oil which possesses very good antioxydant and antymicrobial activities and prevents products from fungial soailage. A significant number of essential oils from fennel, coriander, basil and many others can be used in the same purpose in combination with suitable flavor [1, 11].

Film coating with waxy substances and natural antioxidants such as lignum resin butylhydroxyanisole and others find very good application in active packaging. They protect foods containing fats and oils against oxidation and store them for longer, keeping their nutritional value and safety. Such packs are used for butter, hard cheeses, etc. [2, 7].

Currently, in composition of polymer packages are used enzymes. The implimentation of biologically active packaging materials with immobilised enzymes on the polymeric carrier allows to adjust composition, biological value of food and to intensify the production process. For example, use of lactase to break down milk sugar (lactose) or cholesterolreductase for reducing cholesterol level in milk product.

In film coating are also used enzyme reactive substances in order to remove oxygen. For example glucoseoxydase, ethanoloxydase which are so-called "scavengary".

Quite perspective are the active packages with edible coating. In this case, as a film- forming base are used natural polymers such as polysaccharides (starch derivatives and cellulose) [4]. These substances possess unique properties. The excellent film-forming ability of polysaccharides and their high resolution structure formation are crucial for practice and make them applicable as components of edible coatings, glazing agents and packaging pasty milk, sugar, fruit and vegetable products. These coatings act as humectants protect the food from the loss of mass due to reduce the rate of evaporation of moisture and create a barrier to the penetration of oxygen and other unwanted substances from the environment. When used they slow down the processes of governing decay of food as fat oxydation, protein denaturation and others [7, 10].

Considerating polymeric compounds possess another advantage. They have a high adsorption capacity, which determines their positive physiological effects (adsorbed and dissolved metal ions, radionuclides and other hazardous compounds) when received in the digestive tract of humans [7].

Through the introduction of special additives

from the group of flavors and perfumes, dyes etc. in edible film coating of active packaging, it is possible to regulate the organoleptic qualities of foods - taste, aroma and color. Furthermore, the ability of this layer to retain (immobilize) various compounds allows to enrich products with desirable nutrients such as minerals, vitamins, trace elements complexes, amino acids and others. In this way, is compensated the deficiency of these necessary nutrients for humans [7, 10].

III. Conclusion

The conducted research allows to make the conclusion that the use of food additives in active packaging is essential for extending the shelf-life of products, as well as to improve their organoleptic properties and safety. When applying them is eliminated the necessity of direct use of synthetic chemicals as preservatives in the products. This is an issue for food production which is particularly relevant and significant. Active packaging can contribute not only to prevent microbial spoilage, but also to preserve the biologically active ingredients and nutritional adequacy of the product during storage and transport. They can prevent undesirable physical changes and allow more complete satisfactory reduction of the ever growing demands of consumers. Active packaging can be used for general food packaging, and also in special earmarked.

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ANTIOXIDANT AND ANTIBACTERIAL HERBS

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Abstract. *In cooking, use different supplements (herbs) that improve the taste and aromatic properties of the finished product and at the same time ensuring microbiological safety for a specified retention period. It was found that the tested herbs possess antioxidant and antibacterial properties, and therefore, when added to food, help to increase shelf life, which is especially important when storing food products without a Special cooling, food service in the field.*

Keywords: herbs, antioxidant activity, antimicrobial activity, microbiological safety.

Currently the industry is catering is in the rapid development associated with the expansion of the range of culinary products and increase shelf life. In cooking, use different supplements (herbs) that improve the taste and aromatic properties of the finished product and at the same time ensuring microbiological safety for the required retention period.

The purpose of research - the study of the antioxidant and antimicrobial activity of extracts of these herbs and their mixtures: soriándrum sátivum (coriander), sinnamomum verum (cinnamon), súrcuma (turmeric), zíngiber (ginger), riper nigrum (white pepper), riper (black pepper) , sapsicum frutescens (red pepper), xylopia aethiopica (Moorish pepper), diánthus (cloves), rosmarinus (rosemary), myristica (nutmeg), pimpinélla anísum (anise), piper cubeba (cubeb pepper) and their changes during storage. Investigated the concentration of solids in the extracts was 2,0% and 6,0%, based on the analysis of the formulations used in the preparation of food products [1].

In the preparation of mixtures of herbs used in the most common culinary practice ratios and combinations (Table 1). [3]

Table 1. *Variants recipe blends of herbs*

Name of herbs	Mass fraction in the mixture, wt%.					
	Mixture № 1	Mixture № 2	Mixture № 3	Mixture № 4	Mixture № 5	Mixture № 6
Coriándrum sátivum	10,0	20,0	15,0	5,0	-	5,0
Cinnamomum verum	-	5,0	-	15,0	20,0	-
Cúrcuma	15,0	10,0	10,0	10,0	-	15,0
Zíngiber	15,0	-	20,0	5,0	10,0	-
Piper nigrum	-	20,0	10,0	15,0	5,0	20,0
Píper	5,0	-	-	10,0	5,0	-
Capsicum frutescens	5,0	-	15,0	-	-	-
Xylopia aethiopica	10,0	15,0	5,0	-	10,0	15,0
Diánthus	5,0	-	10,0	15,0	10,0	-
Rosmarinus	5,0	15,0	-	10,0	-	10,0
Myristica	15,0	-	10,0	5,0	10,0	15,0
Pimpinélla anísum	15,0	10,0	5,0	-	15,0	10,0
Piper cubeba	-	5,0	-	10,0	15,0	10,0

Antioxidant content in aqueous extracts of herbs determined amperometric method on the device "Color Jauza-01-AA", which consists in measuring the electric current arising in the oxidation of the substance (or mixture of substances) on the surface of the working electrode at a certain on-potentials

and comparison the received signal with the signal of the standard substance (solutions dihydroquercetin and ascorbic acid), measured under the same conditions. As eluent used 2,2 mM solution of phosphoric acid [2].

The calculation of the antioxidant activity (CA, mg/dm^3) using the formula

$$CA = CA_c \times N,$$

where CA_c – antioxidants found the calibration curve, mg/dm^3;

N – dilution of the test sample.

In analyzing the graphic dependences (Fig. 1) showed that the antioxidant activity of aqueous extracts studied herbs and mixtures depends on the concentration of the extract and its increase from 2,0 to 6,0%, increasing by 1,7 ... 2,0 times. Thus, the greatest antioxidant activity of the studied extracts of herbs has piper cubeba, but the use of this plant is limited because of organoleptic characteristics, and of mixtures of herbs - a mix of № 5.

You can also note that in the course of storage without special cooling (at T=298±0,1 K) antioxidant activity of aqueous extracts of herbs and their mixtures are practically unchanged.

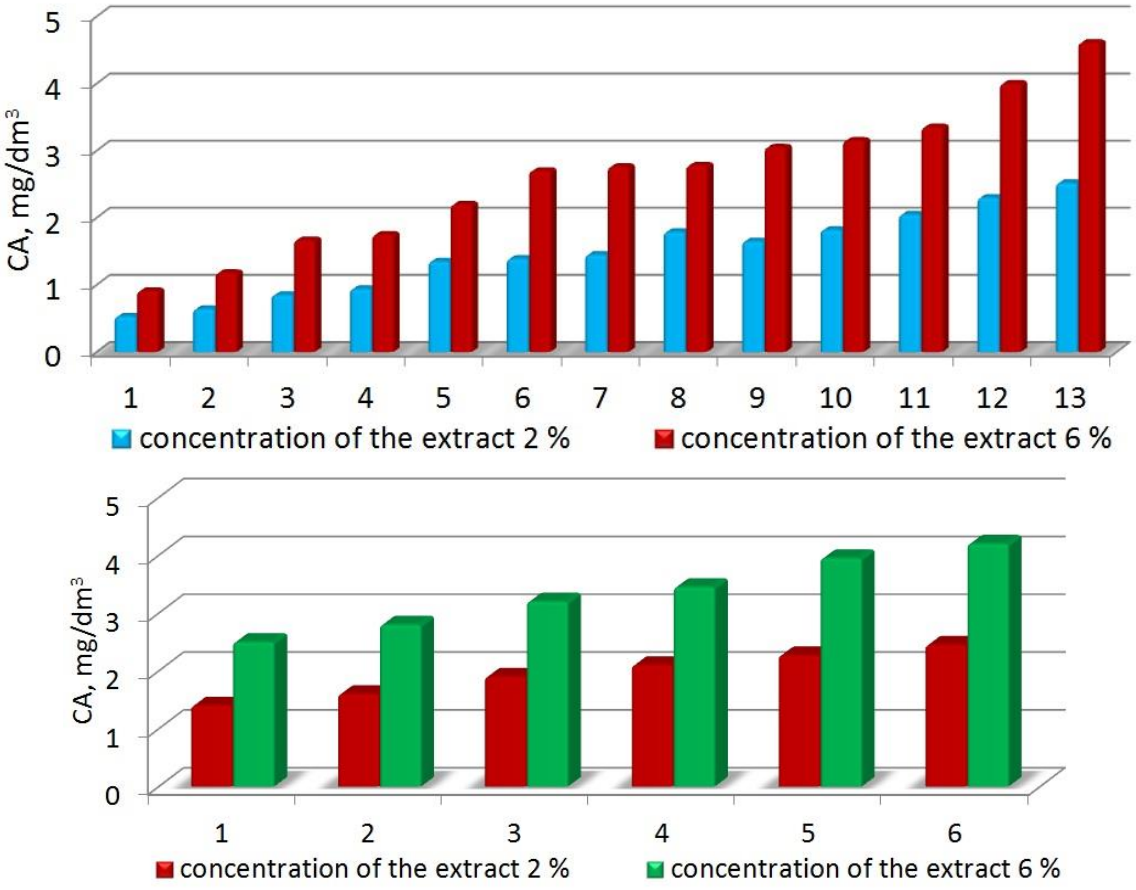

Figure 1. *Antioxidant activity of aqueous extracts of herbs (a): 1 – súrcuma 2 – soriándrum sátivum; 3 – piper nigrum; 4 – zíngiber; 5 – diánthus; 6 – sinnamomum verum; 7 – xylopia aethiopica; 8 – píper; 9 – rosmarinus; 10 – myristica; 11 – pimpinélla anísum; 12 – sapsicum fru-tescens; 13 – piper cubeba and their mixtures (b): 1 – a mix of № 1; 2 – a mix № 2; 3 – a mix of № 6; 4 – a mixture of № 4; 5 – a mixture of № 3; 6 – mixture № 5*

In order to determine the antimicrobial activity of used method for determining the susceptibility of microorganisms to antimicrobial agents, based on the diffusion of antimicrobial agents in the test organisms seeded dense nutrient medium from the wells, around which, after incubation, a zone of growth suppression. Culture medium at the same time provide the growth of micro-organisms and study diffusion of anti-microbial agents, to form the inhibition of bacterial growth.

As dense medium we used a medium L, which includes: yeast extract – 5,0 g, peptone – 15,0 g, NaCl – 5,0 g, agar – 15,0 g, distilled water – 1 liter . As an experienced test cultures using bacteria of the strain Escherichia coli - 113-3 DSM 1900. In the medium made of 15 mm diameter hole in which to place the samples studied aqueous extracts of herbs

to 0,5 cm^3. Crops were incubated at (37 ± 2) °C for 24 hours.

Antibacterial activity was determined by the formation of zones of inhibition of growth contributed to the culture medium Escherihia coli bacteria around the holes with specimens of aqueous extracts of herbs (Tables 2, 3). Served as a control sample antimicrobial drug "Penicillin", in which the diameter of growth inhibition Escherihia coli colonies was 7,5 cm.

Analyzing the data, one can conclude that the antimicrobial activity of the investigated aqueous extracts of herbs depends on the concentration of the extract and its increase from 2% to 6% increase in the 20 - 60%. Found that the greatest antimicrobial activity of the extract has piper cubeba, diameters stunting Escherihia coli colonies which were: d = 5,60 and 7,40 mm – for extracts, with a concentration of 2% and 6%, and the least - an extract súrcuma for which : d = 2,60 and 4,40 mm – for extracts, with a concentration of 2% and 6%.

Table 2. *The diameters of zones of growth inhibition Escherihia coli bacteria in water extracts of herbs*

The concentration of the extract, %	The diameters of the zones of growth inhibition Escherihia coli bacteria in water extracts of herbs, cm												
	Cúrcuma	Coriándrum sátivum	Piper nigrum	Zíngiber	Diánthus	Cinnamomum verum	Xylopia aethiopica	Píper	Rosmarinus	Myristica	Pimpinélla anísum	Capsicum frutescens	Piper cubeba
2 %	2,60	2,90	3,90	4,40	4,50	5,0	5,10	5,20	5,30	5,40	5,45	5,50	5,60
6 %	4,40	4,70	5,80	6,20	6,50	6,80	6,90	7,0	7,10	7,20	7,25	7,30	7,40

Table 3. *The diameters of the zones of growth inhibition Escherihia coli bacteria in water extracts of mixtures of herbs*

The concentration of the etract, %	The diameters of the zones of growth inhibition Escherihia coli bacteria in water extracts of mixtures of herbs, cm					
	Mixture № 1	Mixture № 2	Mixture № 3	Mixture № 4	Mixture № 5	Mixture № 6
2 %	3,40	3,80	5,20	4,70	5,70	4,20
6 %	5,20	5,40	6,50	6,10	7,20	5,70

In the study of antimicrobial activity of extracts of mixtures of herbs found that the greatest antimicrobial activity of the extract is a mixture of № 5, the diameters of stunting Escherihia coli colonies which were: d = 5,70 and 7,20 mm – for extracts, with a concentration of 2% and 6 %, and the least – an extract of a mixture of № 1, for which: d = 3,40 and 5,20 mm – for extracts, with a concentration of 2% and 6%.

Conclusions

Thus, the examined herbs possess antioxidant and antibakterial activity, and, therefore, when added to food, help to increase shelf life, which is especially important for food storage of products without special cooling, the power of the Organization in the field (tourism, expeditions, etc.).

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ON A COMBINATION OF SNR AND THE NUMBER OF FREQUENCY BANDS IN AMPLITUDE AND FREQUENCY MODULATION IN SPEECH PROCESSING STRATEGY IN THE PROCESSING UNIT OF THE COCHLEAR IMPLANT

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Abstract. *The proposed research combines the both amplitude and frequency modulation of speech signals in different conditions in the processing unit of the cochlear implant. The aforementioned combination of both modulations is performed in conditions of different signal to noise ratio and different numbers of the frequency bands parameter. The numeric simulation of the proposed algorithm is used as a base for a comparison between the usage of 1-32 frequency channels in computing the mean squared error and varying the value of the signal to noise ratio.*

Key Words: cochlear implants, amplitude and frequency modulation, speech processing

I. Introduction

Identifying the speaker is not the only thing that is delivered by the acoustic characteristics in speech signals. Some other characteristics allow listeners to get the meaning of the speech but also the speaker's emotion. Previous studies using either naturally produced whispered speech [1] or artificially synthesized speech [2], [3] have isolated and identified several important acoustic cues for speech recognition. For example, computers relying on primarily spectral cues and human cochlear-implant listeners relying on primarily temporal cues can achieve a high level of speech recognition in a quiet environment [4]- [6].

The goal of this study is to show the comparison between the usage of different number of frequency channels in computing the mean squared error and varying the value of the signal to noise ratio and to conclude for the best conditions in the processing unit of the cochlear implant.

A speech signal produced by a male talker is chosen for the purpose. A combination of slowly varying amplitude modulation (AM) and frequency modulation (FM) from a number of frequency bands in speech signals is proposed and testing their relative contributions to speech recognition in electric hearing. The ‘‘slow’’ FM used here tracks gradual changes around a fixed frequency in the subband which is different from previous studies using relatively ‘‘fast’’ FM to track formant changes in speech production [8], [11], or fine structure in speech acoustics [9], [10]. It is evaluated AM only and AM plus FM, and the original unprocessed speech signal in different conditions to compare the results of the processing, and to extract the MSE and the distortion. It is conducted an experiment to test this hypothesis about the relative contribution of the added frequency modulation in the speech signal processing method in the cochlear implants in quiet environment (no masker/noise signal added). The comparison of the AM-only and AM plus FM depending on the number of frequency channels is presented. Than the results of processing by the same algorithm is used to compare the signals in noisy environment.

II. Methods

In this experiment the processed stimuli contain the AM only an the both AM and FM cues. A speech signal produced by a male talker (1,5s.) is used. The main parameters are the number of frequency bands varying from 1 to 32, type of the signal modulation (amplitude modulation only or the both amplitude modulation and frequency modulation) and signal to noise ratio varying from 1 dB to 20 dB.

Thirty-two bands were used to match the number of auditory filters estimated psychophysically over the 80- to 8,800-Hz bandwidth [12].

Fig. 1 shows the block diagram of the processing strategy in general. To produce the AM only or AM plus FM stimuli, a stimulus was first filtered into a number of frequency analysis bands ranging from 1 to 34. The distribution of the cutoff frequencies of the bandpass filters was approximately logarithmic according to the Greenwood map [13]. The band-limited signal was then decomposed by the Hilbert transform into a slowly varying temporal envelope and a relatively fast-varying fine structure [12], [14], [15]. The slowly varying FM component was

derived by removing the center frequency from the instantaneous frequency of the Hilbert fine structure and additionally by limiting the FM rate to 400 Hz and the FM depth to 500 Hz, or the filter's bandwidth, whichever was less [16].

The AM-only stimuli were obtained by modulating the temporal envelope to the subband's center frequency and then summing the modulated subband signals [2], [7]. The AM plus FM stimuli were obtained by additionally frequency modulating each band's center frequency before amplitude modulation and subband summation. Before the subband summation, both the AM and the AM plus FM processed subbands were subjected to the same bandpass filter as the corresponding analysis bandpass filter to prevent crosstalk between bands and the introduction of additional spectral cues produced by frequency modulation. All stimuli were presented at an average root-mean-square level of 65dB (A weighted) with the exception of the SRT measure in Exp. 3, in which the noise was presented at 55 dBA and the signal level was varied adaptively.

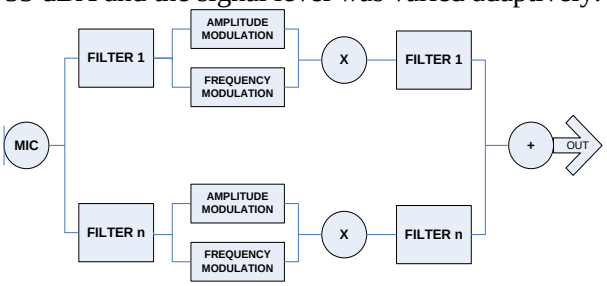

Figure 1. *Signal processing block diagram.*

A signal, s(t), can be approximated by a sum of N band-limited components, x(t), containing both amplitude and frequency modulations`

$$s(t) \approx \sum_{k=1}^{N} x_k(t) = \sum_{k=1}^{N} A_k(t)\cos\left[2\pi f_{ck}t + 2\pi\int_0^t g_k(\tau)d\tau + \theta_k\right] \quad (1)$$

Where $A_k(t)$ and $g_k(t)$ are the k-th band's amplitude and frequency modulations, whereas f_{ck} and θ_k are the k-th band's center frequency and initial phase, respectively.

Fig. 2 shows the block diagram for extraction of AM and FM in the k-th subband. Showed on the top, the AM is extracted by full-wave rectification of the output of the bandpass filter, followed by a low-pass filter LPF 1. The cutoff frequency of LPF 1 controls the maximal AM rate preserved in the AM signal. Additionally, the delay compensation box synchronizes signals between the AM and FM pathways.

The next part of Fig. 2 shows the block diagram for FM extraction in the k-th subband. First, the output of the k-th subband, $x_k(t)$, is subjected to a quadrature oscillator with the center frequency. This manipulation is equivalent to shifting the spectrum of x(k) from f_{ck} to zero and $2f_{ck}$ in the frequency domain. The following low-pass filters (LPF 2 and LPF 2') then extract the slowly varying frequency components (a and b) by removing the high frequency component $2f_{ck}$. In signal processing nomenclature, the slowing-varying components and are termed in-phase and out-of-phase signals of the original subband signal $x_k(t)$, respectively.

Mathematically, if $x_k(t)$ can be described as $x_k(t) = m(t)\cos\left[2\pi f_{ck}t + \varphi(t)\right]$, where m(t) is the amplitude, is the center frequency and is the phase, then the in-phase signal can be derived

$$\begin{aligned} &x_k(t)\times\cos(2\pi f_{ck}t) = \\ &m(t)\cos\left[2\pi f_{ck}t+\varphi(t)\right]\cos(2\pi f_{ck}t) = \\ &\frac{1}{2}m(t)\cos\left[2\pi f_{ck}t + 2\pi f_{ck}t + \varphi(t)\right] + \\ &+\frac{1}{2}m(t)\cos\left[2\pi f_{ck}t+\varphi(t)-2\pi f_{ck}t\right] = \\ &\frac{1}{2}m(t)\cos\left[2(2\pi f_{ck})t+\varphi(t)\right]+\frac{1}{2}m(t)\cos\varphi(t) \end{aligned} \quad (2)$$

Again, the first term in the above equation can be filtered out

$$b = -\frac{1}{2}m(t)\sin\varphi(t) = \frac{1}{2}m(t)\cos\left[\varphi(t)+\frac{\pi}{2}\right] \quad (3)$$

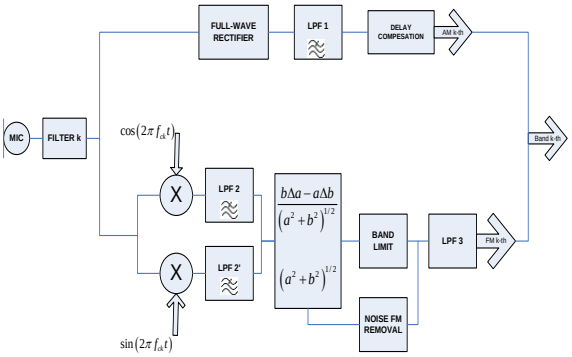

Figure 2. *Amplitude and frequency modulation block diagram.*

Dividing b by a will produce

$$\frac{b}{a} = -\tan\varphi(t)$$

$$\varphi(t) = \tan^{-1}\left(-\frac{a}{b}\right) \quad (4)$$

Finally, the instantaneous frequency can be obtained

$$FM = \frac{1}{2\pi}\frac{d\varphi(t)}{dt} = \frac{d\tan^{-1}\left(-\frac{b}{a}\right)}{2\pi dt} = \frac{b\left(\frac{da}{dt}\right) - a\left(\frac{db}{dt}\right)}{2\pi\left(a^2+b^2\right)} \quad (5)$$

In discrete implementation, differentiation in Eq. (5) can be substituted by calculating the difference in time (Δ) to obtain the slowly varying frequency modulation

$$FM = \frac{b\Delta a - a\Delta b}{2\pi\left(a^2+b^2\right)\times T_s}, \quad (6)$$

where T_s represents sampling period.

MSE is essentially a signal fidelity measure [20]. The goal of a signal fidelity measure is to compare two signals by providing a quantitative score that describes the degree of similarity/fidelity or, conversely, the level of error/distortion between them. Usually, it is assumed that one of the signals is a pristine original, while the other is distorted or contaminated by errors.

Suppose that $x=\{x_i|i=1,2,\ldots,N\}$ and $y=\{y_i|i=1,2,\ldots,N\}$ are two finite- length, discrete signals, original and processed. The MSE between the signals is given by the following Eq. (7).

$$MSE(x,y) = \frac{1}{N}\sum_{i=1}^{N}\left(x_i - y_i\right)^2, \quad (7)$$

where:

N – number of signal samples,

x_i – value of the i[th] sample in x,

y_i – value of the i[th] sample in y.

Signal-to-noise ratio is defined as the power ratio between a signal (meaningful information) and the background noise (unwanted signal):

$$SNR = \frac{P_{signal}}{P_{noise}}, \quad (8)$$

where P is average power. Either signal and noise power must be measured at the same or equivalent points in a system, and within the same system bandwidth. If the signal and the noise are measured across the same impedance, then the SNR can be obtained by calculating the square of the amplitude ratio:

$$SNR = \frac{P_{signal}}{P_{noise}} = \left(\frac{A_{signal}}{A_{noise}}\right)^2, \quad (9)$$

where A is root mean square (RMS) amplitude.

III. Results

Fig.3 shows the results of processing the original signal by amplitude modulation in different conditions- depending on the number of frequency channels (bands) and the SNR.

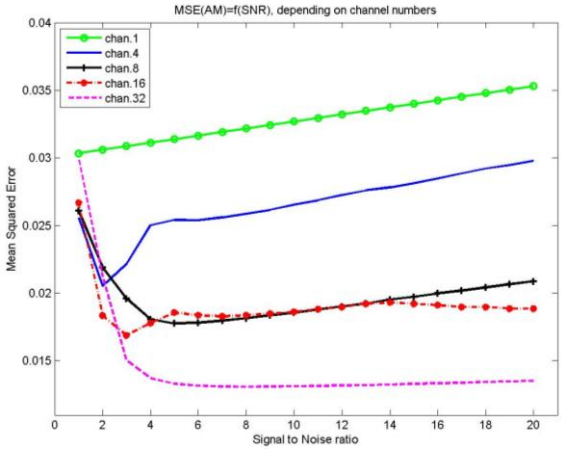

Figure 3. *Diagram of the computed MSE depending on the SNR and the number of the processing bands in AM only conditions.*

Fig.4 shows the results of processing the original signal by amplitude and frequency modulation in different conditions- depending on the number of frequency channels (bands) and the SNR.

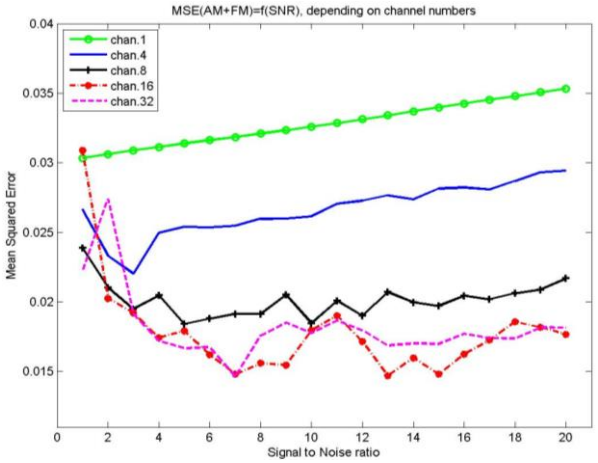

Figure 4. *Diagram of the computed MSE depending on the SNR and the number of the processing bands in AM + FM conditions.*

Fig. 5 shows the diagram of the extracted mean squared error (MSE) depending on the number of the channels and the modulation conditions. We can see the lowermost value of MSE in 32 channels and AM plus FM conditions. We can see again that the MSE is lower in AM plus FM in 8 channels than AM only

condition. Comparing the both cases it is visible the priority of the 32- bands condition in processing the speech signal in AM plus FM.

Table 1. *MSE depending on processing conditions*

Number of bandpass channels	MSE (AM only condition)	MSE (AM + FM condition)
1	0,030337812	0,030338536
4	0,025574211	0,026662412
8	0,026095995	0,023860972
16	0,026680751	0,030894688
32	0,030008300	0,022178849

Table 1 shows the exactly values of MSE which are the base of Fig.5.

The graphical results shows that the comparing between Fig. 3 and Fig. 5 leads to the relation between the number of the processing bands when the noise signal is involved or not included. In the both cases the 32- channel condition is the best parameter for processing the signal because of the final results on the MSE. In Fig. 3 is visible the near values of the MSE in SNR from 4 dB to 20 dB in 32 number of processing frequency bands conditions.

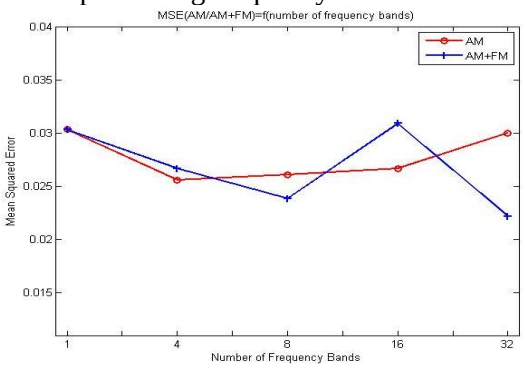

Figure 5. *Diagram showing the results of computing the mean squared error (MSE) depending of the number of the bandpass channels while AM only or AM plus FM condition.*

It is visible in Fig.4 the unstable form of the curves. This unstable form can be expounded in the frequency modulation characteristics. The 32- and 16- number of frequency bands are near to their values. The most stable form of curves in Fig.3 and Fig.4 is the 1- frequency band condition of processing- the worst case.

IV. Discussion

Because the FM cue is derived from phase, the present study argues strongly for the importance of phase information in realistic listening situations. We note that for at least two decades phase has been suggested to play a critical role in human perception [17], yet it has received little attention in the auditory field.

The most direct and immediate implication is to improve signal processing in auditory prostheses. Currently, cochlear implants typically have 12–22 physical electrodes, but a much smaller number of functional channels as measured by speech performance in a quiet environment [18]. The results of our research strongly suggest that frequency modulation in addition to amplitude modulation should be extracted and encoded to improve cochlear implant performance in quiet. Recent perceptual tests have shown that cochlear implant subjects are capable of detecting these slowly varying frequency modulations by electric stimulation [19].

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EMULSIFYING CAPACITY AND EMULSION STABILITY OF ANIMAL LIVER

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Abstract. *Meat food emulsions are liquid multiphase systems consisting of water, fat and emulsifier, liquid being single, relatively stable thermodynamically. Influence the amount of emulsifier emulsifying capacity is proportional and is a growing trend. Emulsion stability after heat treatment increases as the increasing amount of emulsifier (pork liver) is virtually identical to nature addicts emulsifying capacity of the amount of emulsifier. Research has been carried out in model systems representing oil 100ml 100 ml water 7 g liver. On meat emulsion technology (pate, liverwurst) is considered an ideal recipe containing emulsified product: non-fat meat, pig fat or lard, water, the following chemical composition corresponds to 10-12% total protein (including 2 to 2.5 % collagen), 20-25% fat and 60-70% water.*

Key Words: Emulsifying capacity, emulsion stability, liver, protein, fat, water

I. Introduction

To obtain liver products such as pate it is necessary to ensure the smooth (homogene) texture of the finished product which contains soluble and fat-soluble substances.

The purpose of the research was to estimate the emulsifying capacity and emulsion stability of solid liver protein products such as L/A [10].

In general, there can be two types of emulsions, depending of the ratio between two phases

• Water in oil emulsion, the water is dispersed in oil, which is the external phase;

• Oil in water emulsion, the oil is dispersed in water, which is the external phase.

The meat food emulsions are multiphase systems consisting of water, fat and emulsifier, liquid being single, relatively stable thermodynamically. In general, emulsions (single or multiple) have limited stability, where the role of liver protein and emulsifier has the following meat emulsions and products remain steadye as we heat treated condition indicating a high emulsifying capacity so as heat treatment ensuring high stability of the emulsion [1].

Interaction of fat-protein-water type is due to the large number of hydrophilic and hydrophobic groups in proteins. The capacity emulsifying of proteins causes formation of emulsions and their stability.

Hydrophilic and hydrophobic groups of proteins determines the targeting of polar groups to water and non-polar groups to fat. Elastic and strength properties of proteins after heat treatment texture depends on the stability of micelles and, as a consequence, determines the quality of end products(ready-made products). Solubility, the degree of denaturation of proteins, as well as pH and ionic strength (the ionic force) of the solution influence the emulsifying capacity and emulsion stability [2].

The lipid content of pork liver is from 3,6 ... 7,8% and bovine liver from 2,2 ... 3,9% [3].

II. Materials and methods

For the research was used raw material pork and bovine liver from Republic of Moldova refrigerated and thawed after storage. The raw material used to make pate was shredded (minced) to Ø3mm. Emulsion was prepared in microcuter for 8 ... 10 minutes.

The capacity of emulsifying and emulsion stability was investigated on model systems representing oil 100ml +100 ml water +7 g liver [4]. The capacity of emulsifying and emulsion stability are determined by the ratio of emulsified oil to 1 g liver. Emulsion stability is obtained after thermal treatment of liver ($t = 80°C$, $\tau = 30min$). The capacity of retention of fat by absorption [5].

III. Results and discussion

The problem proposed to be solved is determination of lipids (fats) in liver products (pate) as emulsion type L/A. Emulsion stability is ensured by layer (stratum) lipo - protein on cell surface of fat [6].

Emulsifying capacity of the liver and establishment of emulsion based on liver can be achieved on the basis of qualitative and quantitative content of protein and lipids. Capacity of emulsifying and formation of stable emulsions depends on the primary structure of

protein macromolecules, in particular of amino acid residues content with hydrophobic groups [7].

The protein macromolecules composed of polypeptide ionized chain with electric charges and neutral fragments (hydrophobic) shows double capacity, of hydration and of hydrophobic interactions. In complex compositions these macromolecules are oriented and arranged in a determined way. By intensive stirring of food composition protein complexes, water and fat from forming hydrophobic hydration and interaction combined macromolecular structures. On the macromolecules surface protein and on the polypeptide ionized chains is added water. Simultaneously, hydrophobic fragments of the protein macromolecules regects water molecules and adds fragments of the hydrophobic lipid [8].

Compositions consisting of proteins, water and lipids are ordered as follows: polar fragments of proteins bound water, at the same time hydrophobic fragments of protein macromolecules bind lipids by hydrophobic interactions [9]. Therefore lipid molecules are retained by protein macromolecules (Figure 1).

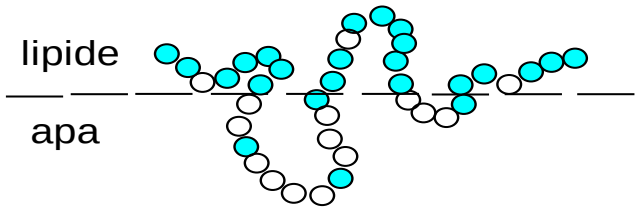

Figure 1. *The scheme of probable orientation of the protein macromolecule phase limit that separates water-protein - lipid.* ○ *- Polar groups of the protein molecule* ● *- the molecule of protein hydrophobic groups.*

By similar retention mechanism of lipids are formed emulsions such as lipid / water. For such systems of power, showing the structure of finished (end) products is important the emulsion stability during of storage (Figure 2).

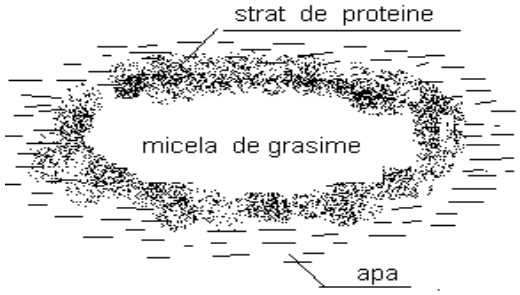

Figure 2. *Schematic representation of emulsion formed by sorption of fat protein micelle on the surface of water-protein - lipid.*

Hydrophobic groups are formed on the outer (exterior) surface of the layer of fat droplets is strongly adsorbed, which acts as a barrier to agglomeration of fat droplets. Hydrophilic groups are oriented to water molecules.

It should be noted that the system's ability to retain the fat meat grows by increasing the amount of collagen. Retention capacity of the protein fat issue is greater than that of muscle and liver protein, which is explained primarily by the fact that collagen protein shell swells considerably during heat treatment and is able to retain fat in its cells. To improve the emulsifying capacity and therefore the amount of fat linked protein emulsion preparations is used protein preparations in particular soy protein isolate.

The capacity of emulsifying the liver is determined by its protein emulsifying ability. Proteins are surface active substances are characterized by a polar amino acid ratio (hydrophilic) and non-polar (hydrophobic), following which actually become able to reduce surface tension at the boundary of phase separation A / L. Surface activity of emulsifiers polimoleculari, primarily determined by differences and spatial structure of content quantity, location and availability of polar and non-polar groups of the protein molecule. System fat / water phase boundary separating the proteins in Brownian motion account open so that non-polar amino acids are oriented lipid phase and polar amino acids in the aqueous phase, where it is their interaction. At a subsequent absorption of protein molecules they interact not only with the phase of fat and water, but also with other proteins that contribute to formation of a strong gel layer.

The orientation of hydrophilic protein groups to the aqueous phase and to those hydrophobic lipid phase separation limit as adsorbed layer, decreases the surface tension in the disperse systems and makes them resistant to aggregation and at the same time ensures smooth(homogeneous) texture.

From total oil that is introduced into the system oil + water + liver in emulsion stage contains 46,8 ... 57,3% of the total oil 200ml, remaining oil forms separated lipid phase of water and liver.

The indicator values of emulsifying capacity and emulsion stability of oil composition, water, bovine liver are higher on average by 2 ... 4%, this is mainly due to a non-polar amino acids ratio in polar amino acids in bovine liver is greater (0, 72) as pork liver (0,70).

Table 1. *The capacity of emulsifying and emulsion liver stability*

Indicator	unit of measurement	pork liver	beef liver
The emulsifying capacity	g mulsion / 1g liver	6,7… 7,7	7,0… 8,2
The emulsion stability	g mulsion / 1g liver	4,5… 5,2	5,0… 5,6
Fat retention capacity	% of the initial mass	17,7… 21,9	19,8… 23,4

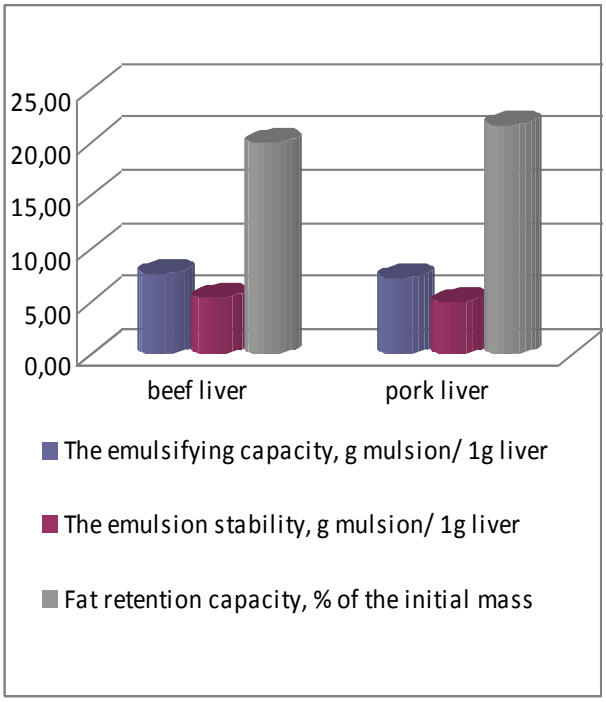

Figure 3. *Emulsifying capacity and fat retention capacity of bovine and pork liver.*

Following the heat treatment of oil-based emulsion / water / protein (porcine liver) compared to 100: 100: 7, emulsion stability is 1,5 to 1,6 times less practical as emulsifying ability of proteins of porcine liver which shows that, in the heat treatment, liver proteins have the ability to stabilize the emulsion lower than when using the native liver.

IV. Conclusions

1. Emulsifying capacity is greater than the emulsion stability on the grounds that the denaturing the liver proteins lose this ability.

2. Emulsifying capacity and emulsion stability depends on the ratio hydrophilic amino acids in the liver protein macromolecules structure.

3. Liver emulsifies from 7,2 to 7,6 parts oil to one part liver and as a stabilizer after heat treatment stabilizes from 4,8 to 5,3 parts oil to one part liver.

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PORK AND BOVINE LIVER IMPORTANT SOURCES OF NUTRIENTS

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Abstract. *This study includes the methodology of obtaining the functional products with high nutritional value. Nutritional quality of food is determined by: the content and quality of carbohydrates, proteins and lipids, content of soluble and fat soluble vitamins, mineral content and, as well, the content of biologically active substances. Tis article includes a study use of pork and beef liver to diversification range production. It was found that the nutritional indicators as VN10, CS, EV in liver are almost identical to those in meat, in some cases even higher, being influenced by many factors: variety, age, anatomical part, animal's nutrition. It was found that liver is an important raw material in terms of nutritional quality and can be used to diversify the products with the optimized values. It showed the correlation nutritional indicators as VN10, CS, EV in liver based products.*

Key Words: nutritional value, pork liver, bovine liver, nutritional indicators VN10, CS, EV

I. Introduction

Nutritional quality of food is determined by: the content and quality of carbohydrates, proteins and lipids, content of soluble and fat soluble vitamins, mineral content and, as well, the content of biologically active substances [1, 11].

This paper present the evaluation, based on bibliographic and experimental study of the nutritional value of meat and liver of bovines/porcinis. Was studied the chemical composition and nutritional value of raw materials. Nutrition index (VN_{10}), chemical score (CS) and the energy value was calculated. It was found that bovine and porcine liver are interesting in terms of nutrition, economics, serving as raw material for food diversification with improved nutritional value.

II. Materials and methods

Tested products: swine meat, swine liver, bovine meat, bovine liver purchased from supermarket.

The study of nutritional value was conducted on the bibliographical study of the chemical composition of products mentioned above [1-11].

The assessing of VN10 in tested samples was carried out in the formula suggested by the nutritionist F. Strimska [9, 10].

The energy value of food (100g) is achieved by the relation:

$$VE = 4.1 \times (\%\mathrm{Pr}) + 9.3 \times (\%L) + 4.1 \times (\%G), Kcal/100g \quad (1)$$

Where:

Pr, L and C are the percentage content of proteins, lipids and carbohydrates in food.

The evaluation of protein quality was performed using methods standardized and approved by the International Committee of FAO and WHO. In tested products were assessed the chemical index (CS – chemical score), according to the formula [9, 11], using the Excel program.

The interdependence between the indexes VN10, CS, VE was determined through Excel program, using the Pearson function (r^2).

III. Results and discussion

In Figures 1 and 2 shows average content of protein, fat, ash meat and liver of bovine/pork.

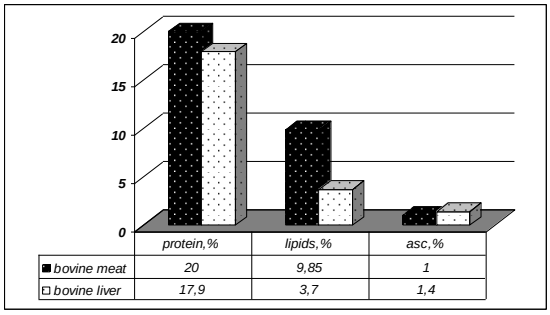

Figure 1. *The chemical composition of bovine meat and bovine liver*

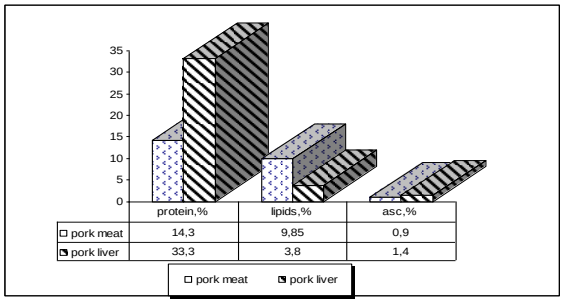

Figure 2. *The chemical composition of pork meat and pork liver*

The data presented in table 1 and table 2 shows that meat and liver represent important sources of macro and micronutrients [5-10]. The nutritional value of liver is almost identical to the meats, except the content of essential amino acids, B vitamin complex and the content of I_2 and Fe, where the liver is a leader [1 – 4, 9-11].

Table 1. *The content of nutrients in the chemical composition of products*

Macro and micro nutrients	Bovine		Pork		Standard Protein FAO/ WHO g/100g protein
	meat	liver	meat	liver	
Protein, g/100g	20,0	17,9	14,3	18,8	-
Lipids, g/100g	9,8	3,7	33,3	3,8	-
Carbohydrate, g/100g	1,0	5,3	0,9	4,7	-
Ca, mg/100g	10	9	7	9	-
P, mg/100g	200	314	164	347	-
Fe, mg/100g	2,9	6,9	1,7	20,2	-
Vitamin A, mg/100g	traces	9.2	traces	3.45	-
Vitamin B_1, mg/100g	0,07	0,3	0,52	0,3	-
Vitamin B_2, mg/100g	0,18	2,19	0,14	2,18	-
Vitamin C, mg/100g	traces	33	traces	21	-
Valine	1,15	1,25	1,14	1,25	5
Isoleucine	0,94	0,93	0,97	1,00	4
Leucine	1,62	1,59	1,54	1,76	7
Lysine	1,74	1,43	1,63	1,49	5.5
Methionine + Cysteine	0,90	0,756	0,76	0,77	3.5
Threonie	0,88	0,81	0,96	0,92	4
Triptophan	0,27	0,24	0,27	0,31	1
Fenilamină + Tyrosine	1,70	1,66	1,51	1,68	6
Total essential amino acids	9,20	8,67	8,77	9,28	36

Table 2. *The VN10, CS, VE indicators and Pearson correlation of meat and liver of swine and bovine*

Product analyzed	**VN_{10}**	**VE, kcal / 100g**	**CS, %**	**Pearson correlation**
Bovine meat	20,19	124,55	9,2	VN_{10} = f (CS); r = 0,51954
Bovine liver	59,46	328,91	8,7	VN_{10} = f (VE) r = 0,41829
Pork meat	19,27	339,27	7,7	VN_{10} = f (VE) r = 0,635524
Pork liver	56,69	117,34	9,4	VN_{10} = f (VE) r = 0,44812

IV. Conclusions

In this paper we have realized a bibliographic study of chemical composition of meat and liver of swine and bovine, from which it was found that the meat and liver presents important sources of macro and micro nutrients, in some cases the content of such elements as Fe, I_2, essential amino acids is higher in liver than in meat.

It was found that the nutritional indicators as VN_{10}, CS, EV in liver are almost identical to those in meat, in some cases even higher, being influenced by many factors: variety, age, anatomical part, animal's nutrition.

It was found that liver is an important raw material in terms of nutritional quality and can be used to diversify the products with the optimized values.

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WALNUT MEAL COMPOSITION AND ITS USE

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Abstract. *This paper includes a bibliographic study of the chemical composition of walnut meal. Also there are presented various ways to use the meal. There are characterized the benefits that can be obtained using the meal at bread baking. In the meal, obtained by cold pressing of Juglans regia L. walnuts were determined the total oil content, total acidity and total water content. They baked bread samples with and without walnut meal. In control and research bread samples there were appreciated following indicators: bread porosity, acid value, specific volume. It was found that the introduction of walnut meal in bread composition led to obtaining a quality product with relevant nutritional value and pleasant sensory properties. It is important to mention that the shelf life of the samples with introduced meal was comparatively higher than those of controls. Microbiological stability of work samples has been comparatively higher than those of control.*

Key Words: walnut meal, ways for using meal, baking bread meal

I. Introduction

Walnut Juglans regia L is a species of great importance across Central Asia, Europe and SUA. Walnuts are collected for home consumption, sold at local roadside stands and markets. Walnut trees are further utilized for their high/quality timber to make a wide array of products. The leaves, barks and other plant parts are used for medicinal remedies; trees are grown and maintained for soil conservation purposes [8].

Currently, nut orchards area in Moldova is 12,000 hectares. Annually, about 80 percent of production is exported to 40 countries, including Italy, France, Germany, England etc. [16-18].

Walnut's fruit has a remarkable nutritional composition. Walnut contains quinones, oils, tannins. Nuts contain essential fatty acids, including cis-linoleic and alfa-linolenic acid. The kernels contain oil, mucilage, albumin, muneral matter, cellulose and water. Walnut is considered a good source of dietary minerals. Potassium, phosphorus, magnesium and iron are found in significant quantitities in these nuts [2].

Walnut are nutrient-rich food due to high contents af fats, proteins, vitamins and minerals. They are also good sources of flavonoids, sterols, pectic substances, phenolic acids and polyphenols [1, 7]. Walnuts have a special value in dietary food, resulted from their carbohydrate content (11-14%), proteins (14-16%) and represented of the essential aminoacids and lipids (62-65%), of which 44-48% are polyunsaturated fatty acids [11].

Therefore, the walnut is classified as a strategic species for human nutrition and is included in the FAO list of priority plants [10]. The part of the fruit (kernel) is consumed fresh, toasted or mixed with other confectionaries [9].

The major component of walnut oil are triacylglycerols (980 g/kg oil), which are monounsaturated FAS (mainly oleic acid) and polyunsaturated FAS (PUAS, linoleic and linolenic acid). Oil contents reported by Juglans regia L. leaves have been used mostly in worldwide traditional medicines as antimicrobial, antihelmitic, astrigent, keratolytic, antidiarrhoeal, hypoglyceaemic, depurative, tonic, diabetes and asthma etc. [3, 6, 8].

Proteins are major and essential components of healthy and wholesome food. In solving the problem of giving population food proteins, it is essential the integrated and rational use of them, contained in plant materials or to prepare food based on proteins. One type of such raw materials is walnuts.

Integrated use of walnuts as a source of edible oil, food and protein, will create full, rich in protein foods, significantly cheaper, compared to products from the expensive animal protein.

After nut oil extraction it is obtained the meal, which is 30-47% depending on the used method for oil extraction: cold pressing, hot pressing, solvent extraction and so on [12]. Composition of walnut meal obtained from extraction of oil is less studied. Usually nut meal is used as animal feed.

We aimed to evaluate the chemical composition of the meal and find ways to use as an ingredient in various food compositions. This paper is the first research study of the chemical composition of walnut meal Juglans regia L., nutritive value, the

benefits which can provide the oil industry waste materials, and ways to use it in food.

It was found that the introduction of walnut meal in bread composition led to obtaining a qualitative product with relevant nutritional value and pleasant sensory properties. It is important to mention that the shelf life of the samples with introduced meal was comparatively higher than those of controls. Microbiological stability of work samples has been comparatively higher than those of control.

II. Materials and methods

2.1. Plant material

Walnuts Juglans regia L. were collected manually in October 2011 in Chisinau, the center of Moldova. Were dried and stored them at room temperature. Walnut oil is obtained by cold pressing. Meal was collected and served as research object.

There were introduced different proportions in baking dough composition to study how to use the meal as a partial substitute for flour. However, taking into account the chemical composition of the meal rich in protein, amino acids, minerals, vitamins, antioxidants we can obtain a product with a relevant nutritional value.

2.2. Chemicals and reagents

Other materials used for bread baking: wheat flour, wate , salt, yeast.

Bread quality control and research (with added walnuts meal) was determined by standard metods.

The following indicators were tested:

- *Porosity*, as GOST 5669-96.Bread. Method for determining the porosity.
- *Organoleptic indicators and product weight*, as GOST 5667-65. Bread and bakery products. Rules for receiving, sampling methods, methods for determining the organoleptic indicators and weights of products.
- A*cidity*, as GOST 5670-96. Bread. Methods for determining the acidity.
- *Humidity*, as GOST 21094-75. Bread and bakery products. Methods for determining the moisture.

III. Results and discussion

In addition, nuts have significant economic and medicinal value to human health because of their biochemical composition of polyunsaturated fatty acids, especially 18:2 and 18:3 and protein value [12]. Well, they contain other beneficial components such as vegetable proteins (e.g., arginine, leucine), carbohydrates (e.g., dietary fiber), vitamins (e.g., vitamin A, E), pectic substances, minerals (magnesium, potassium, phosphorus, sulfur, copper and iron), plant sterols and phytochemicals [4, 12].

Walnut meal proteins contain a lot of amino acids such as histidine, trionina, tryptophan, valine, isoleucine, leucine [13-15].

After pressing, it is got the cake (meal) rich in proteins, in which there are a lot of essential amino acids, especially lysine. According to the content of the latter (6.2g per 100g) walnut proteins are superior to egg proteins. Nuts contain minerals as potassium, calcium, iron, cobalt salts, phosphorus and sulfur. Especially a lot of them are potassium, phosphorus and sulfur. From trace elements in the fruit should note the presence of iodine and zinc [6, 7].

Study [1, 14] shows that meal chemical composition of coconut is rich in remarkable quantities of complex amino acid proteins, essential fatty acids, minerals, flavonoids and dietary fibers, which are sources of micro flora healthy bowel, improve digestion and eliminate toxins from the body (Figure 1).

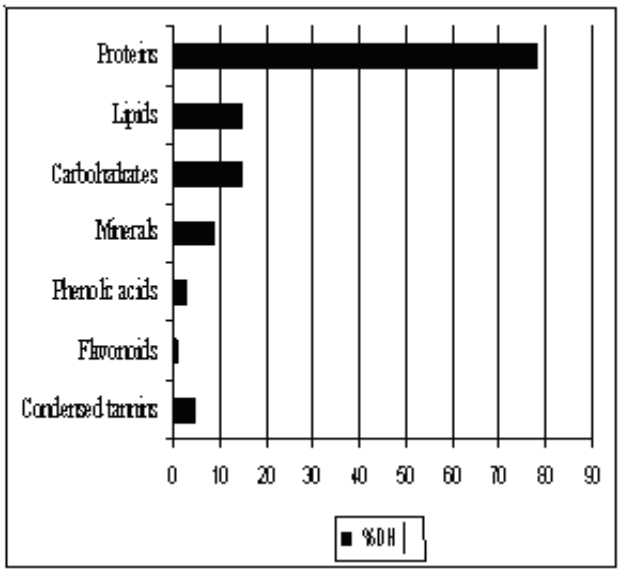

Figure. 1. *Chemical composition of coconut meal*

Meal is contained in 100g of 26% of the daily dose of vitamin B_6, vitamin B_1, 23%, 25% - folic acid, 34%, phosphorus, 79% - copper and manganese. Nut flour has a rich source of polyphenols and other compounds endowed with antioxidant properties. Meal nut has a rich source of proteins and dietary fibers which can be used to increase the nutritional value of foods such as: bakery products, various houses, sauces etc.

Quality indicators are presented in the table 3.1. of walnut meal: sensory, physicochemical and microbiological indicators.

Table 3.1. *Quality indicators of walnut meal*

Indicators	**Characteristic**	
Sensory	Pieces or fine yellow powder-brown. Not contain foreign particles or additives and impurities. Specific taste of walnut products. Contains no aromatic additives.	
Physicochemical	Structure	Pieces or powder, not containing compressed blocks.
	Granulation	Min 99% passing through sieve 300 microns / HD
	Moisture	9% max (GOST13979.1-68)
	Protein	34 % min (N2x6.25)
	Fat	10 % max (GOST 13979.2-94)
	Ash	7% max (ash, 10% HCl insoluble < 1%, GOST 13979.6)
	Microscopity	Opaque particles (IF/RH) are missing.
Microbiological	Aerobic microbiological quantity	10.000/g max
	Yeasts and molds	100/g max
	Coliforms	10/gmax
	E. coli	no
	Salmonella	No/25g

Walnut meal is added to the dough along with the flour in amounts of 2.5, 5 to 10%. The research results have been established as the quality of the finished product and depend on the amount of admixture (Table 2).

The ratio estimation of height / diameter of bread sample showed that this indicator is directly proportional to the amount of used cake (maximum value was 0.56 when meal was replaced with 10% flour. Indicator for the control sample was value of 0.42.

Thus, it was found that when using walnut meal bread volume increases from 1 to 3.66%, the best results were obtained when using meal of 2.5%.

Table 3.2. *Physicochemical indicators of the quality of bread with the addition of walnut meal*

Quality indicators	**Control sample**	**Content meal in used bread, %**		
		2,5	**5**	**10**
Specific volume of bread, cm^3	690,26	715,53	692,77	696,6
Ratio height / diameter	0,42	0,47	0,52	0,56
Core porosity, %	76,47	77,91	78,85	75,40
Humidity core, %	41,0	44,4	42,1	41,5
Core acidity, degree of acidity	2,8	3,0	3,1	3,2

IV. Conclusions

Using recycled materials in the production of walnut oil solve rational use of food resources by non waste technology.

Walnut meal is a rich source of biological active substances such as protein composition of essential amino acids, polyunsaturated fatty acids, mineral compounds, flavonoids and dietary fiber.

Chemical composition of walnut meal showed prospects of their application as a source of protein and dietary fiber to increase the biological value of bakery products.

It was found that the quality of finished bakery products depends on the amount of nut meal introduced together with flour.

Study carried out showed that the best results of bread quality indicators are achieved when amounts of 5% flour are used.

It was found that the introduction of walnut meal in bread composition led to obtaining a qualitative product with relevant nutritional value and pleasant sensory properties.

It is important to mention that the shelf life of the samples with introduced meal was comparatively higher than those of controls. Microbiological stability of work samples has been comparatively higher than those of control.

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MICROBIOLOGICAL RISK ESTIMATION AT WALNUTS LONG-TERM STORAGE

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Abstract. *The paper presents a bibliographic and experimental study of the microbiological incidence of Juglans regia L. walnuts at long-term storage. It was effectuated the microbiological control of autochthonous walnuts stored at different ambient conditions. We found that an improper storage leads to an increased risk of nuts infection with various microorganisms such as fungi, bacteria and acaridae. It is important to mention that the walnuts are vulnerable to fungi attack with Aspergillus flavus and A. parasiticus, species that produce aflatoxins, considered to be carcinogenic. Other isolated fungi in walnuts are: Alternarea, Penicillium, Phoma, Botryosphaeria, Fusarium, Cladosporium, etc. Microorganisms which are presented in walnuts may produce lipolytic enzymes that degrade the product quality and safety; also the same impact can be reflected on other foods based on these fruits. There are many factors that have a great influence on walnuts quality and safety. The most important are climatic conditions, varieties, cultivation methods and storage methods.*

Key Words: microbial contamination, risk, long-term storage, fungi attack, walnuts quality and safety

I. Introduction

Various nuts are used as a raw material in many industries as well as for a direct consumption. Walnuts Juglans regia L. are rich sources of unsaturated fatty acids (56.2%, Ω3, Ω6, Ω9), proteins (15-25.2 %), carbohydrates (0.2 -15%), vitamins (B_1, B_2, B_3, B_6, B_9, C, E, PP, β-carotene, A), minerals (Fe, K, Ca, Mg, Na, S, P, Cl, I_2, Co, Mn, Cu, Zn) etc. [8]. They contain an important amount of proteins and fat and their products have wide acceptance as food throughout the world. Due to the extremely high fat, proteins and low water content (3.8 - 4.5) of various nuts such as hazelnut, almonds, walnuts, these products are quite refractory to spoilage by bacteria. Molds can grow upon them if they are stored under conditions that permit sufficient moisture for their propagation [6].

Nuts quality is determined by the content of unsaturated fatty acids, proteins, vitamins, minerals, antioxidants and the presence and activity of enzymes [5, 11-13]. Microbial incidence reduces nuts quality and safety [1, 11-13]. Microbial stability of nut varieties, cultivated in Moldova, is less studied. In this context we present a bibliographic an experimental study of Juglans regia L. walnuts.

II. Materials and methods

2.1. Plant material

The study was conducted on autochthonous walnuts Juglans regia L., harvested in Telenesti, the central part of Moldova, during 2010 and 2011. Walnuts were purchased from commercial network and stored at room temperature (25°C). Microbiological control was conducted on 100 walnuts in each year.

2.2. Microbiological analyses

A twenty-five g analytical unit taken from the 100g sample unit was used in aerobic bacterial count, mold count. Plating for total bacterial count was carried on standard nutrient agar and for molds on Sabouraud dextrose agar [10, 13]. There were studied isolated colonies after incubation period of 3 days at 28°C for bacterial count and 7 days at the same temperature for mold count.

Also, total mold, yeast contamination and bacterial counts of the samples were determined using a dilution plate method. About 25g walnut samples (whole nuts, kernels) were suspended in 225ml of 0.1% peptone solution and homogenized. Homogenized samples were diluted with 0.1 % peptone solution to concentrations of 10^{-2} and 10^{-3}. Each dilution (100µl) was dispensed and spread onto sterile BA/Sabouraud in Petri plates, followed by incubation at 28ºC for 3/5 days. Total mold and yeast counts were estimated by back-calculation based on observed mold for each dilution and was expressed as the number of colony forming units (CFU) per gram in each sample.

III. Results and discussion

Bibliographic study indicates that the nuts microbiota depends on variety, geographical conditions, climate, collection, processing and storage [1-13]. The climatic parameters (temperature, relative humidity, precipitations and UV irradiation), the composition of leaf surface (senescence) and the availability of nutrients change over the microbial colonization [1]. Molds of many genera may be found on examined nuts [5]. The mould genera, occurring most frequently in shelled and whole nuts, were: *Aspergillus, Penicillium, Rhizopus, Mucor and Cladosporium.* Aspergillus and Penicillium species predominated in all tested shelled nuts being (32-39%) *Aspergillus* and (25 to 31%) *Penicillium, w*hile *Aspergillus* was dominant in all the whole nuts (41-50%). Other genera were found to be in low percentage (*Rhizopus, Mucor and Cladosporium*). The results [13] are in accordance with Smith and Arend [6] who stated that *Aspergillus, Penicillium, Rhizopus, Mucor and Cladosporium* represented the common genera in nuts.

Data reported by M.J. Sejiny et al. [5] show the qualitative and quantitative distribution of different groups of bacteria present in the shelled and whole nuts. It was found that Gram positive spores forming bacilli, Gram positive Micrococcus, Gram positive rods and Gram negative short rods generally contaminated the samples. Their quantitative distribution varied through the tested nuts. The most dominant bacteria appearing in the whole nuts were Gram positive spores forming bacilli being 55% and 68% in shelled and whole walnuts respectively, while Gram positive Micrococcus were 24% and 29% respectively.

Walnut trees are a habitat for a wide variety of fungal and (to a lesser extent) bacterial taxa some of which can pose a serious threat to plant health. Measures should be taken further to prevent the accumulation of microbial inoculums, e.g. removal of leaf debris [1]. Microbial biodiversity associated with the walnut Juglans regia L. is reported in several studies [1-7, 13]. Study [1] includes microflora analysis on walnuts trees in South Tyrol (Italy). From a total of 3.880 isolated cultures, the wide majority of the isolates (3.742) belonged to fungi (96.4%); only 138 (3.6%) were bacteria. Fungal isolates were classified intro 30 genera, *Alternarea, Penicillium, Phoma, Botryosphaeria, Fusarium, Cladosporium, Phyllosticta and Epicoccum being the most taxa.* The most isolates were obtained from leaves 45.3% than from twigs 31.8% and fruit 23.0%. Microbial growth depends on variety of environmental parameters. Among them temperature and relative humidity play a predominant role [5, 6].

Infestation rate of walnuts samples Juglans Regia L., stored during one year and two years, was different. The results of the investigation show that the nuts storage led to an increased microbial infestation in nearly 25-30 % of analyzed nuts.

The results of microbiological control of walnut Juglans regia L., grown in Moldova are the limits reported in bibliographic study. It was found that longer storage of nuts contributes to an increased microbial infestation. The rate of infestation abundance of walnuts harvested in 2010 amounted to fungi and yeasts 50% and 30% Gram positive and Gram negative 20%. Nuts harvested in 2011 were less infested. Heavy infestation rate being only 10% for fungi, yeasts, Gram positive and Gram negative bacteria.

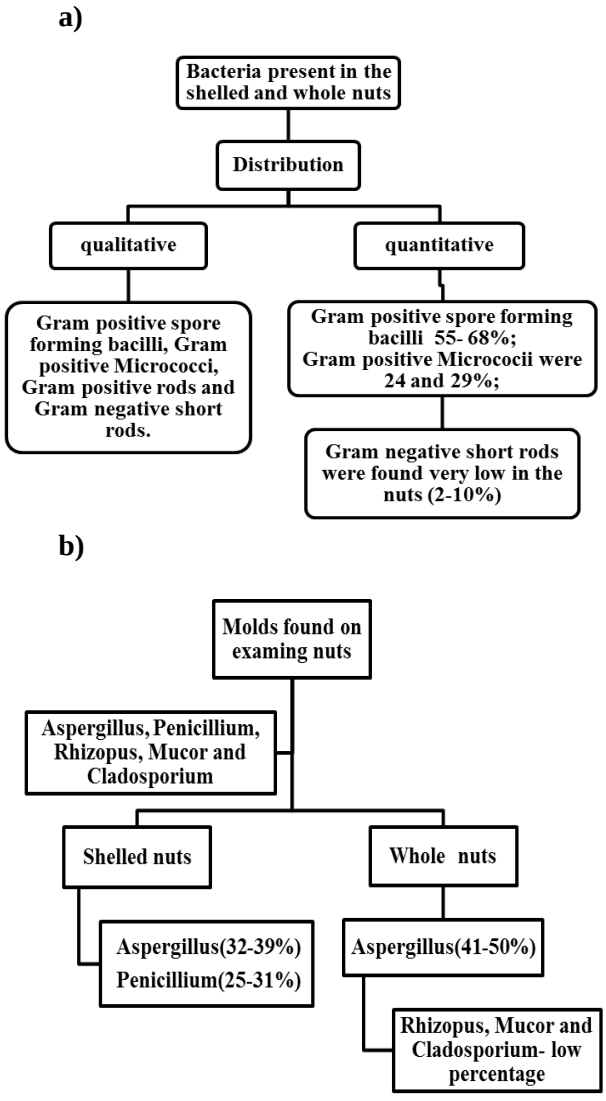

Figure 1. *Microbial contamination of Juglans regia L walnuts stored in Moldova*
a) Contamination with bacteria, b) Contamination with molds

In Figure 2 we present some images of colonies of microorganisms found in walnuts Juglans regia L., collected in Moldova, stored 22 months at temperature 25°C.

Figure 2. *Isolated colonies after incubation period of 7 days at 28°C*

The data obtained show that shelled walnuts prevailed infestation of fungi g. *Aspergillus* and *g. Penicillium* and the nuts were shelled frequently isolated molds *g. Aspergillus*. The article also aims at qualitative distribution of bacteria isolated nuts investigated, which formed following sequence: **Gram-positive bacilli > Micrococcus Gram positive > Gram-negative bacilli.**

Producing Quality Walnuts Food [9] denotes: walnut growers can minimize the potential for food borne illness outbreaks resulting from crop contamination by following good management practices. Potential for contamination of walnuts with these organisms is highest during harvest when the nuts are dropped to the ground. Procedures that can be applied to minimize potential for on-farm contamination of walnuts:

- Irrigation and Water Quality considerations
- Nutrient application
- Harvest
- Good manufacturing practices in the plan
- Proper storage.

IV. Conclusions

Bibliographic and experimental study shows that walnuts Juglans regia L. can be infected with fungi, yeasts and bacteria, which minimize their quality. Infection rate depends on climatic parameters (temperature, relative humidity, precipitations and UV irradiation), variety nuts and storage conditions. Good storage practices should be implemented to minimize the levels of insects and fungi in storage facilities.

To store walnuts should be taken into account Code of Practice for the prevention and reduction of aflatoxins contamination in nuts (CAC/RCP 59-2005) [3].

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TOWARD IMPROVING THE HEAT-STABILITY OF EXISTENT FRUIT FILLINGS BY USING GELLAN GUM

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Abstract. *With the aim of meeting the demand for new bakery and confectionery products in domestic and international markets, a novel approach for heat-stable fruit fillings development was proposed.*

The novel formulations for heat-stable fruit fillings with the addition of gellan gum were developed within a large range of soluble solids (from 40 to 70 °Brix) using Response Surface Methodology (RSM) and the quality parameters of the fillings were investigated.

The fruit fillings' samples were prepared locally from apple puree, sugar, low acyl gellan gum and citric acid. The manufactured fruit fillings after a short-term storage were put through standard bakery test to evaluate their heat-stability after baking process performed under exactly fixed conditions: at a temperature of 220°C for 10 minutes.

Regression polynomial equation and response surface plots were used to determine the interaction effects of two main factors – soluble solids and percentage of gellan gum – on heat-stability of fruit fillings.

Key Words: fruit filling, gellan gum, heat-stability, design

I. Introduction

At the present time one of the major challenges related to the filled bakery products manufacturing (doughnuts, muffins, puff pastries, croissants, pies and buns) consists in thermal instability (physicochemical and sensory degradation, water releasing, otherwise called "syneresis", etc.) of fruit compositions used as fillings in these products.

Many water-rich fruit fillings release water not only during baking but also before and after it (for example, during the freeze-thawing processes or long-term storage). Water release also can happen if filled pastry product is frozen after being baked (precooked frozen food) and then thawed out when used. This water exuding evokes a long series of disadvantages, both from the aesthetic and organoleptic point of view, which make the final product unacceptable for the consumer because of his low quality. Heat-instable fruit filling is like a dampish fruit stuffing for pastry that releases an excess of moisture wetting the dough and preventing it from the correct baking (the pastry does not raise uniformly, having inside part raw and wet, while its outside part tends to dry or burn). The melting behavior of fruit fillings depends on baking duration and temperature in the oven. Fruit compositions start melting and flow if they are exposed for a short time to a temperature much higher than their melting point or if they undergo high temperatures in the range of the melting point in a long time. In order to produce heat-stable fruit fillings with high quality characteristics, the melting temperature of fruit half-stuff composition has to be higher than the temperature in the oven.

The heat-instability of fruit fillings that is evident during baking process at high temperatures in the oven can be improved by using special carbohydrate polymers with stabilizing properties such as gellan gum.

The aim of this study was to develop a novel technology of natural heat-stable fruit filling's manufacturing on the basis of experimental design technique. The introduction of gellan gum in fruit filling's composition was carried out in order to improve the thermo-stability of the final product, while maintaining its high quality (sensory, physicochemical and textural) characteristics before, during and after baking process.

Carbohydrate polymers, such as gellan gum, play essential role as functional ingredients in many food desserts including puddings, mousses, jams, jellies and fillings. One of the most important functions of these food polymers is to bind a large amount of water. Preliminary information about water holding capacity of carbohydrate polymers is significant in practical applications, such as heat-stable fruit fillings. Thus, not every carbohydrate polymer can be used in heat-stable fruit fillings formulations due to different water-binding capacity: water release can occur when the food products prepared on the basis of carbohydrate polymers are subjected to external

forces, temperature fluctuation such as freeze-thawing or as a result of passive diffusion (syneresis), mainly after long-time storage. Water release may result in changing texture, form and volume and reducing quality. Thus, water-binding capacity is the most important criterion in selection and evaluating the acceptability of food stabilizers for heat-stable fruit fillings development.

Gellan gum as a novel polysaccharide gelling agent is worth to be studied in the development of heat-stable fruit fillings, because can form both elastic and brittle heat-stable gels, depending on cation concentrations. Gellan gels are soft and easily deformable below the critical calcium concentration, and brittle above. Textural properties of gellan-based gels are related to water binding capacity and the microstructure.

The major objectives of the research were to establish the optimum percentage of gellan gum and its impact on heat-stable, rheological and sensory properties of the fruit fillings developed within a wide range of soluble solids – from 40 to 70 °Brix (or % sugar).

Response surface methodology (RSM) as one of the most commonly used techniques of experimental design [1, 2] was applied for predicting the optimal quantity of gellan gum added to fruit filling's composition for attributing high thermo-stability to the final product.

II. Materials and methods

2.1. Raw materials

Sugar was purchased at a local supermarket (Chisinau, Republic of Moldova). Apple aseptic puree was manufactured at the canning plant "Conserv-E" (Chisinau, Republic of Moldova). Citric acid solution (50%) was prepared locally in the Laboratory of Functional Foods of the Practical Scientific Institute of Horticulture and Food Industry (Republic of Moldova). Low acyl gellan gum powder (KELCOGEL F) was acquired from the Moscow International Exhibition for Food Ingredients, Additives and Flavorings – "Ingredients Russia" (Moscow, Russian Federation).

2.2. Sample preparation

The fruit fillings samples were produced locally from apple puree (12 °Brix), sugar, low acyl gellan gum powder (KELCOGEL F) and citric acid (50% concentration). Citric acid was added to the fruit fillings compositions not only to provide the right flavor, but also to prolong the shelf life of the finished product. Ascorbic acid (otherwise called Vitamin C) as a well known naturally occurring organic compound with strong antioxidant properties can also be used in combination with citric acid to increase biological value of the fruit fillings. The amount of added sugar was dependent on the final required soluble solids for each fruit filling sample.

First of all, the selected gellan gum was dissolved with a part of the sugar in the ratio 1:10 in the hot distilled water using a high speed stirrer. The dispersion was heated from 95 to 98°C and the temperature maintained for 1 min to give clear solutions. The total amount of the sugar was initially divided into two parts, and the second one was introduced to the smooth apple puree, and heated till the sucrose has dissolved. The remaining ingredients were then added, resulting in lower pH and full dissolution of gellan gum. With the initiation of gelling, mixing time became critical, and agitation was only continuing for one to two minutes. A longer time could have break the gel as it began to form, resulting in a soft, useless sludge.

The fruit fillings were hot bottled in glass bottles properly sterilized, leaving a space for the formation of vacuum after closing. After cooling, the flasks were sealed and stored for two days in the refrigerator for subsequent sensory and physicochemical analyses.

2.3. Physicochemical and sensory analysis

The physicochemical and sensory analysis of the finished fruit fillings were conducted at the Laboratory of Functional Foods of the Practical Scientific Institute of Horticulture and Food Industry of the Republic of Moldova.

The soluble solids of the prepared fruit fillings were measured using benchtop refractometer ABBE and expressed in °Brix. The pH was determined by dint of potentiometric method, introducing the electrode directly into the fruit fillings.

The fruit fillings samples (including control samples) were presented to 10 randomly selected members of the panel before and after baking. The panelists evaluated samples for color, taste, aroma, texture and overall acceptability, using a 5-point hedonic scale where 5 denoted 'dislike extremely' and 1 – 'like extremely'.

2.4. Determination of heat-stability

After short-time storage in the refrigerator at 4ºC, the sterilized fruit fillings were put through a standard bakery test to evaluate their thermal stability as follows: a specific amount of prepared fruit filling was given into a base of special filter paper named "Blue ribbon" with a diameter of 120 mm by a metal ring with defined geometry (50 mm diameter and 10 mm height) and then was baked under exactly fixed conditions: at a temperature of

and 220°C for 10 minutes [3]. During and after this baking process all changes in physicochemical, textural and sensory attributes of the tested fruit fillings were evaluated. The bakery index was determined by measuring the sample diameter before and after baking established by placing a line across the sample and calculating by using the following formula:

$$BI = 100 - \frac{D_2 - D_1}{D_2} \cdot 100, \qquad (1)$$

where:

BI – bakery index, %;

D_1 – average diameter of sample before baking, mm;

D_2 – average diameter of sample after baking, mm.

Diameter of the filling sample before baking is 50 mm, because it's the diameter of the metal ring used in bakery test. For measuring the sample diameter depending on its shape from two to four lines were drawn, and the average was calculated.

For validation experiments we used not only the filter paper type "Blue ribbon", but also pastry samples with a diameter of 60 mm. For the pastry samples we selected another metal ring with the following dimensions: 30 mm diameter and 10 mm height.

2.5. Statistical analysis

Response surface methodology (RSM) was used to establish the optimal quantity of gellan gum added to fruit filling's composition for attributing high thermo-stability to the final product. Therefore, as independent variables there were selected: the quantity of gellan gum and the soluble solids content. The levels of these variables were set at: 0.1 (or "-1" in coded form) and 1.0 (or "+1" in coded form) for percentage of gellan gum and 40 (or "-1" in coded form) and 70 (or "+1" in coded form) for soluble solids content, °Brix. The heat-stability of fruit fillings as a response variable was expressed through the bakery index (BI, units). All experiments adjusted by the design planned in coded and encoded form of process variables, were conducted randomly. The results obtained through application of response surface methodology were verified by conducting the validation experiments under the optimized conditions of all the factors. The adequacy of the regression equations was evaluated by the F-test for analysis of variance (ANOVA) using statistical package STATISTICA v.6. 3D surface plots were drawn by using MATCAD v.15 to investigate the effect of all the factors on the response variable.

III. Results and discussion

Initially and after baking at a temperature of and 220°C for 10 minutes the fruit filling samples were evaluated for physicochemical characteristics and sensory quality. All samples of the fruit fillings prepared different ratio between soluble solids content and gellan gum had a low pH (from 3.35 to 3.6). The statistical analysis of sensory scores revealed that no significant difference was found ($p>0.05$) for the taste, flavor and overall acceptability of the fruit fillings with low soluble solids (40-50 °Brix) and medium percentage of gellan gum (0.5-0.8) gellan gum before and after baking. However, sensory score of listed parameters for the fruit fillings with high soluble solids (60-70 °Brix) and low content of gellan gum (<0.5) was significantly different from initial values after baking process ($p<0.05$). Mean values ranged from 4,5 to 5,0; 4,0 to 5,0; 4,5 to 5.0; 3,5 to 5,0 and 4,0 to 5,0 for color, taste, aroma, texture and overall acceptability respectively indicating good acceptability of the product.

Physicochemical and sensory characteristics of the fruit fillings analyzed under laboratory conditions have demonstrated that they meet the international food standard CODEX STAN 296-2009 FOR JAMS, JELLIES AND MARMALADES.

The development of heat-stable fruit fillings was carried out by using design expert software package STATISTICA v.6. The experimental design with different independent variables in coded and encoded form i.e. soluble solids and gellan gum content and bakery index as response is reported in Table 1.

Table 1. *2^k design matrix for heat-stable fruit fillings development on the basis of low acyl gellan gum*

№ exp.	X_1 gellan gum content, %		X_2 soluble solids, °Brix		Y bakery index expressing thermal stability
	Coded values	Encoded values	Coded values	Encoded values	
1	1	1	-1	40	96,15
2	1	1	1	70	40,0
3	-1	0.1	-1	40	50,0
4	-1	0.1	1	70	30,0

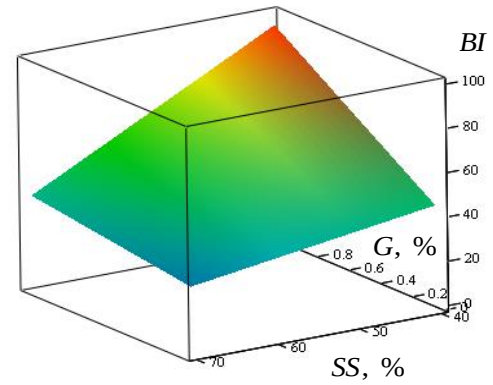
After experimental data processing, the following regression equation describing fruit fillings heat-stability in terms of actual values was obtained:

$$BI = 66.18 + 104.83 \cdot G - 0.53 \cdot SS - 1.34 \cdot SS \cdot G \quad (2)$$

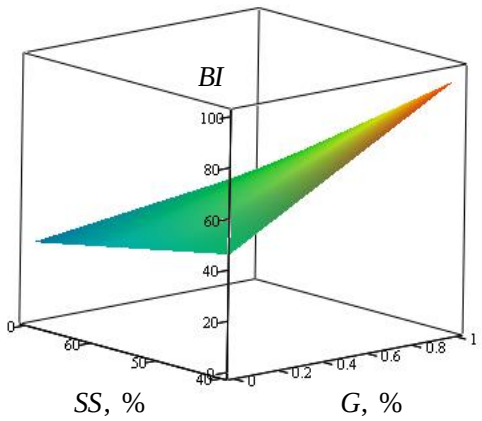
where
BI – bakery index, units;
G – gellan gum content, %;
SS – soluble solids, %.

F-test for analysis of variance (ANOVA) using statistical package STATISTICA v.6 has shown that the model was statistically significant. The experimental data values of the validation experiments closely agreed to the predicted values of developed model with acceptable percentage error.

The response surface plots of the polynomial equation represented above have been plotted using MATCAD v.15 as a function of two variables i.e. soluble solids and gellan gum content (Figure 1) in order to investigate their common effect on the response variable – bakery index.



a)



b)

Figure 1. *3D surface plots: Effect of gellan gum concentration and soluble solids on bakery index*

Judging from the validation experiments, it was evident that the fruit fillings samples prepared with high content of gellan gum (>0.7%) didn't become runny, burnt or caramelized and stayed well on its place, maintaining its original shape and volume.

It also didn't make the biscuit sample under and around it wet, meaning that the gellan gum retained all free water in the filling during baking. The consistency of the fruit filling after baking was nice, smooth but not sticky or gooey. The taste and aroma of the fresh apple fruit puree was also well preserved.

IV. Conclusions

Application of the Response Surface Methodology (RSM) for the optimization of traditional bakery fruit fillings' formulations through using gellan gum lead to the development of heat-stable fruit fillings with high quality characteristics.

According to the present investigation it was established that low acyl gellan gum would be more advantageous to use in fruit fillings compositions with low or medium soluble solids content (40-50 °Brix) in order to improve their heat stability, while maintaining high sensory and textural characteristics.

There was obtained the final regression equation in terms of actual factors in order to describe the influence of soluble solids and gellan gum content on fruit filling's heat-stable properties.

The adequacy of the regression equation evaluated by the F-test for analysis of variance using statistical package STATISTICA v.6 has shown that the model was statistically significant.

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