Effect of cholesterol-lowering starter cultures in smoked sausages on the formation of bioactive peptides and lipid profile in triton-induced hyperlipidemic rats

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**Abstract.** Three types of sausages were studied: without starter cultures; with experimental starter cultures from Moscow State University of Food Production collection; with starter culture Bactoferm SM 194 (Chr. Hansen). No pathogenic bacteria in any sample were revealed. According to the results of PCR-RT, the number of lactic acid bacteria in all samples was about the same - 1×10⁹-2×10⁹ CFU/g. T-RFLP analysis shows the maximum number of lactobacilli sausages with experimental starter cultures averaged 69.59% of the total microbiota. The study of the protein profile of raw smoked sausages showed changes in protein fractions and presumably formation of biologically active peptides. A wide range of peptide mass peaks, with certain differences, was obtained by mass spectrometry. Feces of rats (groups 1-5) were studied by T-RFLP. The proportion of lactobacilli was 2.09%, 2.65% and 2.35% in groups 3-5 respectively. The serum atherogenic index did not differ significantly between the groups due to the low content of non-LDL and non-HDL cholesterol in control rats compared to the other groups. The greatest decrease of serum cholesterol concentration was measured in rats that consumed sausages with experimental starter cultures, mainly due to almost 3-fold (P<0.05) decrease in cholesterol of low-density lipoproteins compared with the control.

1. Introduction

The proteome of meat and its by-products is a good source of not only essential nutrients, but also bioactive sequences inhibiting angiotensin-converting enzyme (ACE), or with antioxidant, opioid, immunomodulatory, prebiotic, mineral-binding, cholesterol-lowering and antimicrobial activity [1-7]. The possibility of forming various bioactive peptides through microbial proteolysis has been studied in recent years [8,9]. Thus, some lactic acid bacteria, such as *Lactococcus lactis* and *Lactobacillus helveticus*, generate bioactive peptides during fermentation. This system consists of a number of different intracellular peptidases, including endopeptidases, aminopeptidases, dipeptidases, and tripeptidases [10-11]. However, proteolytic enzymes released by lactic acid bacteria are very different according to the species and strains, and therefore, generated bioactive peptides from these bacteria can belong to different groups [12,13].
Sanz et al. [14] evaluated the activity of proteases and aminopeptidases of *Lactobacillus casei* CRL 705 on muscle proteins. Proteinase activity of whole cells caused degradation of a large number of sarcoplasmic proteins; partial hydrolysis was also associated with cell extracts. Peptide profiles were strongly changed, and more significant generation of free amino acids was noted, when whole cells were combined with cell extracts. Basso et al. [15] compared the proteolytic activities of *Lactobacillus sakei* DSM 6333, *Lactobacillus plantarum* B21 and *Lactobacillus farciminis* DSM 201 84 on meat sarcoplasmic proteins. All strains demonstrated proteolytic activity, especially against muscle glycogenphosphorylase isoform and glyceraldehyde-3-phosphate dehydrogenase.

These studies presented data on the individual effects of starter cultures on the proteolytic changes of meat product raw materials. Of course, the hydrolytic effect of starter cultures, individually or in combination, on the protein profile of meat products is obvious. In our current work, we evaluate the effect of starter culture compositions on the supposed formation of biologically active peptides during fermented sausages processing. It was also previously found that combined starter cultures reduced cholesterol more extensively than single strains [16,17].

The influence of fermented sausages on the fecal microflora and lipid profile of rats with a model of Triton-induced hyperlipidemia was evaluated.

### 2. Materials and Methods

Raw smoked sausages contained beef, horse meat, beef fat, soy protein and spices and were processed by Ekopro (Ivanteyevka, Moscow region). The technology consisted of the following stages: freezing of meat raw materials at -1 to -3 °C, homogenization in a cutter, filling the cases with minced meat, fermentation at 3°C for 5-7 days, smoking at 13-15 °C for 2-3 days and drying for 5-7 days at 15 °C. Smoking and drying processes were repeated two or three times until the product ready. Three types of sausages were processed: BSK – without starter cultures; SKK – with starter cultures from Moscow State University of Food Production (MSUFP) collection (*Lactobacillus sakei* 104 (B-8906), *Pediococcus pentosaceus* 28 (B-8888) and *Staphylococcus carnosus* 108 (B-8953)); SKXX – with starter culture Bactoferm SM 194 Chr. Hansen (*Pediococcus pentosaceus*, *Staphylococcus carnosus*, *Staphylococcus xylosus*, *Lactobacillus sakei* and *Debaryomyces Hansenii*).

T-RFLP was used to analyze the microbiota of raw smoked sausages. PCR amplification of 16S bacterial rRNA genes was performed using 63F primers labeled at the 5'-end (D4-WellRed fluorophore) and 1492R (Bigle LLC, Russia). Amplified fragments were isolated on agarose gel, and then restriction of DNA amplicons from the reaction mixture was conducted. Samples were precipitated, dissolved in SLS Sample Loading Solution (BeckmanCoulter, USA) with the addition of 600 BP molecular weight marker (BeckmanCoulter, USA) and separated by capillary electrophoresis with fluorescence detection using an automatic sequencer CEQ8000 (BeckmanCoulter, USA). Calculation of peak sizes and areas was performed using the FragmentAnalysis software (University of Idaho, USA).

The total number of microorganisms and lactobacilli in sausages was determined by PCR-RT, using a set of reagents for PCR-RT with Taq DNA-polymerase and enzyme activity inhibiting antibodies in the presence of the dye EVAGreen (LLC NPO DNA-Technology, Russia) according to the manufacturer’s recommendations.

Two-dimensional electrophoresis (2DE) was performed according to the method of O’Farrell with isoelectric focusing in ampholine pH gradient (IEF-PAGE). Subsequent detection of the proteins was carried out by staining with silver nitrate (Panreac, Spain). Protein fractions were excised from the gel, ground and submitted to trypsinolysis (Sigma, Germany). The peptides obtained were investigated by MALDI-TOF MS and MS/MS mass spectrometry on an Ultraflex MALDI-TOF mass spectrometer (Bruker, Germany) with UV laser (336 nm) in the positive ion mode and in the molecular weight range of 500-8000 Da with calibration according to known peaks of trypsin autolysis. Mass spectra of tryptic peptides were analyzed using the Mascot program.

T-RFLP-analysis of animal fecel microbiota included the following stages: separation of the total DNA of microorganisms; PCR amplification of bacteria gene fragments (16S rDNA) with
fluorescence primers (usually the 5' end) and 63F CAGGCCTAACACATGCAAGTC and 1087R TACGGHTACCTTGTACGACTT (Bigle LLC, Russia); enzymatic treatment of amplificate using endonucleases HaeIII, HhaI and MspI according to recommendations of the manufacturer (Fermentas, Lithuania) (usually the restriction enzyme that recognizes a sequence of four nucleotides was used); separation of restricted DNA fragments in a polyacrylamide gel in a sequencer CEQ 8000 (Beckman Coulter, USA) together with a fluorescent DNA marker of known size, Standart-600 (Beckman Coulter, USA). The sequencer was equipped with a computer program for automatic calculation of fragment length – Fragment Analysis (Beckman Coulter, USA), based on the comparison of electrophoretic mobility of fragments of each sample with the sample length standards. Each peak in T-RFLP-grams corresponded to one type of microorganism, while the fluorescence intensity of the peak describes its percentage in the microbial community. Determination of the phylogenetic origin of the microorganisms was performed using the programs and databases FragSort (University of Idaho, CIIIA) http://mica.ibest.uidaho.edu/trflp.php.

The study of cholesterol-lowering effects of raw smoked sausages was carried out on Triton-induced hyperlipidemic rats. Mature Wistar male rats (220±5 g) were formed in statistical groups (n=1-5=10) by randomization according to body weight. Previously, during 20 days, group 3 animals were administered BSK, group 4 – SKXX, and group 5 – SKK. All sausages were mixed with standard chow. Group 1 consisted of intact rats (n=10), group 2 of control animals kept under equivalent conditions. On day 21, Triton X-100 (previously dissolved in a physiological solution) was injected intraperitoneally at a dose of 300 mg/kg of weight to rats in groups 2-5 (n=40), while group 1 (n=10) animals were injected intraperitoneally with an equivalent volume of sterile water. On day 22, the animals were euthanized (VETtech, UK), and blood samples for biochemical studies were taken.

Biochemical investigations were carried out on an automatic analyzer BioChem FC-360 (HTI, USA) according to instructions applied to measurement kits (HTI, USA). Total cholesterol (TCL), triglyceride (TG), cholesterol low-density lipoproteins (CL LDL) and cholesterol high-density lipoproteins (CL HDL) levels were measured in rat sera. Atherogenic index was calculated by the following formula: (AI) = (TCL - CL HDL)/ CL HDL.

STATISTICA 10.0 software was used in this study for the statistical analyses. The results were calculated as mean ± standard error (M±SE). Significant differences were tested by one-way ANOVA, followed by Tukey’s test. Differences with P-values less than 0.05 were considered as statistically significant.

3. Results and Discussion
Pathogenic microorganisms were not detected in any of the experimental sausages. According to results of T-RFLP, the maximum number of lactobacilli occurred in the SKK sausage and averaged 69.59% of the total microbial population, in BSK – 66.40%, in SKXX – 57.90%; coefficient of variation was less than 5%. According to PCR-RT, the number of lactic acid microorganisms in all sausages was almost the same, 1×10^9 CFU/g in BSK and SKK, and 2×10^9 CFU/g in SKXX. The large population of lactic acid microorganisms in BSK detected by T-RFLP and PCR-RT could be explained by development of spontaneous lactic microbiota, which was not necessarily homofermentative and could negatively influence the quality of fermented meat products. Staphylococci were also found, subsequently identified to strain level. Thus, the microbiota of all raw smoked sausages was non-pathogenic, mostly lactic acid bacteria and uncultivated bacteria.

2DE of sausages showed that starter cultures from the MSUF collection retarded the formation of N- and C- terminal fragments of myosin heavy and light chains; actomyosin complex decomposed more slowly compared with commercial starter culture, but a number of enzymes, such as muscle creatin phosphokinase and enolase, were degraded faster by aldolase A. Electrophoregrams of BSK sausage confirmed the degradation of protein fractions.

A wide range of peptide mass peaks in the studied sausages was obtained by mass spectrometry, but there were some differences between treatments. BSK sausage was characterized by the widest mass spectrum (m/z 500-5000) and a large number of mass peaks (23 peaks). The number of mass peaks
reduced to 20 in SKK and was mainly in the range of m/z 500-3500. The spread of mass peaks was decreased, especially in peaks with m/z >2500. The total number of peaks reduced to 19 in SKXX, and a portion of the peaks moved to m/z 800-1800. Specific biologically active peptides should be identified during further studies (Figure 1).

![Mass spectra of raw smoked sausage peptides](image)

**Figure 1.** Mass spectra of raw smoked sausage peptides

The effect of the diet containing raw smoked sausages with starter cultures on bacterial fecal community composition of experimental animals was studied by T-RFLP analysis (Table 1).

**Table 1.** The content of microorganisms in the faeces of experimental animals, %

| Microorganisms                  | Group 1     | Group 2     | Group 3     | Group 4     | Group 5     |
|---------------------------------|-------------|-------------|-------------|-------------|-------------|
| Normal fecal microorganisms     |             |             |             |             |             |
| Cellulolytic bacteria,          | 42.65±1.35  | 54.12±1.66  | 39.10±1.21  | 68.39±2.10  | 35.33±0.98  |
| including:                      |             |             |             |             |             |
| *Bacteroidetes* spp.            | 0.75±0.54   | 7.43±0.74   | 5.62±0.03   | 5.72±0.10   | 2.27±0.49   |
| *Clostridiaceae* spp.           | 0.44±0.22   | 7.52±0.39   | 11.14±0.74  | 21.63±1.04  | 10.88±0.92  |
| *Ruminococcaceae* spp.          | 16.01±0.90  | 18.56±0.85  | 11.18±0.86  | 12.77±0.78  | 8.11±0.81   |
| *Eubacteriaceae* spp.           | 20.99±1.10  | 18.75±0.91  | 8.00±0.39   | 24.82±1.27  | 11.60±0.87  |
The percentages of cellulolytic bacteria, lactobacilli and *Selenomonadales* spp. in the animal feces were high. The percentage of lactobacilli in groups 3-5 was the highest and amounted to 2.09%, 2.65% and 2.35% of the total population, respectively, perhaps because diet of animals in these groups included raw smoked sausage with lactic acid bacteria. In feces of group 1 experimental animals, bifidobacteria were not detected. The greatest population of bacilli (5.72%) was found in the feces of group 5 rats. Staphylococci were found in all groups. The proportion of *Selenomonadales* spp. in all feces was high, except in feces of group 4 rats (1.33%). The content of actinobacteria and enterobacteria in all feces was approximately the same, except feces of group 3 rats, in which the percentage of actinobacteria was 6.70%.

The presence of pathogenic microorganisms was observed in the rat feces: *Campylobacter* was not detected in feces of animals from groups 1, 4 and 5; peptococci were found in small quantities in all feces. The fusobacteria content was slightly increased in groups 1 and 2 compared with the other rat groups. *Pasteurella* was found only in rat faeces from group 2 and amounted to 0.97% of the fecal population. Among transitory microflora associated with food, *Pseudomonas* were identified. Uncultivated bacteria were observed in all feces, the percentages of which ranged from 12.38% to 33.56%.

The analysis of changes in serum lipid profile of experimental animals 24 h after Triton X-100 injection revealed that in control animals (group 2), the content of TCL significantly increased by 70.0% (P<0.05) compared with intact rats (group 1), mainly due to an increase in CL LDL by 3.9-fold (P<0.05), but HDL CL also increased by 46.6% (P<0.05), and the concentration of TG did not change statistically significantly in the experimental groups. The greatest decrease of TCL was observed in group 5 (SKK) and amounted to 32.0% (P<0.05) compared with the control (group 2), mainly due to a decrease in CL LDL by almost 3-fold (P<0.05). However, serum AI of animals that consumed experimental sausages did not differ significantly from the control (group 2) due to the low content of CL non-LDL and non-HDL in control rats, which was quite high compared with the other groups (Table 2). Thus, it was shown that sausages with starter cultures from the MSUFP collection demonstrated a cholesterol-lowering effect.

| Lachnospiraceae spp. | 4.46±0.66 | 1.86±0.15 | 3.16±0.24 | 3.45±0.17 | 2.47±0.42 |
| Lactobacillales spp.  | 0.74±0.17 | 1.88±0.17 | 2.09±0.21 | 2.65±0.15 | 2.35±0.31 |
| Bifidobacteriales spp.| 0.00±0.10 | 0.09±0.04 | 0.27±0.07 | 0.39±0.04 | 4.23±0.56 |
| Bacillales spp.       | 2.65±0.68 | 3.27±0.22 | 2.77±0.35 | 2.09±0.53 | 5.72±0.67 |
| Selenomonadales spp.  | 21.75±1.08| 7.36±0.60 | 18.50±0.97| 1.33±0.24 | 10.38±0.84 |
| Potentially pathogenic microorganisms |
| Enterobacteriaceae spp. | 0.13±0.05 | 0.42±0.08 | 0.21±0.08 | 1.62±0.17 | 0.64±0.09 |
| Actinobacteria spp.   | 3.18±0.65 | 3.70±0.15 | 6.70±0.88 | 3.65±0.55 | 4.73±0.61 |
| Staphylococcus spp.   | 0.22±0.10 | 0.13±0.07 | 0.37±0.11 | 0.11±0.02 | 0.46±0.07 |
| Fusobacteria spp.     | 6.23±0.22 | 4.85±0.32 | 3.15±0.56 | 2.69±0.41 | 2.04±0.24 |
| Peptococcaceae spp.   | 2.33±0.17 | 2.92±0.17 | 0.35±0.12 | 2.50±0.26 | 0.39±0.11 |
| Pasteurellaceae spp.  | 0.00      | 0.97±0.07 | 0.00    | 0.00     | 0.00     |
| Campylobacteriaceae spp. | 0.00      | 0.13±0.10 | 0.10±0.07 | 0.00     | 0.00     |
| Transitory and uncultivated microorganisms |
| Pseudomonadaceae spp. | 0.93±0.59 | 2.58±0.10 | 0.05±0.14 | 2.20±0.60 | 0.17±0.10 |
| Uncultivated microflora | 19.19±0.94| 17.58±0.75| 26.34±1.10| 12.38±0.55| 33.56±1.32|
Gallego et al. [18] described proteolysis of three traditional European dry fermented sausages from Spain, Italy and Belgium, as well as peptide and free amino acid profiles. Their obtained data was explained by differences in composition, conditions of processing and starter culture used in each type of sausage. Moreover, the combined action of muscle and microbial enzymes in these products contributed to the formation of bioactive peptides, demonstrating ACE-inhibitory properties and antioxidant activity. Spanish and Belgian fermented sausages showed the maximum values of ACE inhibition activity, while the Belgian sample showed the highest antioxidant inhibitory activity against the 2,2-diphenyl-1-picrylhydrazyl radical.

Elevated cholesterol level in serum is associated with risk of cardiovascular diseases. Cholesterol increases lipid peroxidation, protein oxidation and the production of free radicals, impairs the antioxidant system (SOD, CAT, GPx and GSH), as well as the activity of ATPase and causes histopathological disorders. Bioactive peptides can be considered as a tool for the prevention or treatment of these disorders. Peptides, identified as cholesterol-lowering, include lactostatin (IIAEK), enterostatin (VPDPR), peptides DPR, LPYP and LPLPR [19]. Peptides of this type are expected to demonstrate different mechanisms of action and can increase the excretion of bile acids with feces, can bond with phospholipids, and can display cholesterol-lowering activity.

Currently, bioactive peptides with proven cholesterol-lowering activity are obtained from various sources [19-21]. Thus, L. helveticus KIII13, isolated from fermented cow’s milk and producing bioactive tripeptides isoleucyn-proline-proline and valine-proline-proline, significantly reduced serum cholesterol and LDL levels in mice with induced atherogenic hypercholesterolemia [20]. Similarly, administration of L. plantarum 14 per os to mice C57/BL6 that were fed high fat diet led to decrease in the mass of adipose tissue, serum cholesterol and leptin; significant changes in weight gain and concentrations of conjugated linoleic acid in serum were not observed [21].

Table 2. Serum lipid profiles of experimental animals

| Parameter | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|-----------|---------|---------|---------|---------|---------|
| TCL, mmol/L | 2.13±0.03* | 3.62±0.15* | 2.78±0.13* | 2.48±0.11* | 2.46±0.14* |
| TG, mmol/L | 0.53±0.03* | 0.47±0.03 | 0.56±0.05 | 0.49±0.04 | 0.55±0.05 |
| CL LDL, mmol/L | 0.53±0.01* | 2.05±0.08* | 0.85±0.04* | 0.79±0.03* | 0.69±0.05** |
| CL HDL, mmol/L | 1.03±0.01* | 1.51±0.06 | 1.09±0.07* | 0.99±0.03* | 1.04±0.05* |
| CL non-LDL and non-HDL | 0.60±0.02* | 0.07±0.01* | 0.84±0.08** | 0.71±0.07* | 0.72±0.05* |
| AI | 1.10±0.03 | 1.41±0.06* | 1.57±0.08* | 1.51±0.07* | 1.35±0.05* |

* – significant difference compared with group 1 (P<0.05), ** – significant difference compared with group 2.

In a number of studies, it was noted that the addition of protein hydrolysates and biologically active peptides of animal origin to the diet of rats with high cholesterol can play a role in reducing atherogenic parameters [22-24]. Dietary additives with kimchi powder or L. plantarum in fermented sausages are effective in reducing the level of lipids, cholesterol and atherogenic index in rat serum. The concentrations of triglycerides in serum of experimental animals that consumed fermented sausage with kimchi and L. plantarum were not significantly different compared with the control group. The levels of total cholesterol, low-density lipoproteins and high-density lipoproteins in the serum of rats that consumed sausage with kimchi were significantly lower than in the control. The level of free cholesterol in serum and atherogenic index of rats that consumed sausage with kimchi and L. plantarum were significantly lower than in the control [22]. Drotningsvik et al. [23] studied the influence of marine fish protein hydrolysates containing peptides on typical markers of metabolic disorders in the model of obesity and diabetes of fa/fa Zucker rats [23]. It was shown that diets containing hydrolyzed herring or salmon proteins can affect growth, lipid metabolism, regulation of glucose levels after meals and the composition of fatty (mono- and polyunsaturated) acids in serum.
and adipose tissue in rats with a model of metabolic syndrome. Protein hydrolysates of zebra blenny, containing biologically active peptides with antioxidant activity, recover disorders caused by the biochemical histopathological effect of cholesterol in rats with hypercholesterolemia [24].

4. Conclusions
The results obtained expand the knowledge of proteolysis, which occurs during processing of sausages fermented with starter cultures. Their potential as natural sources of biologically active peptides is evident and gives additional value to these products, which are still not well enough studied. Specific peptide sequences responsible for the observed biological activities should be identified during further research. Consumption of raw smoked sausages with cholesterol-lowering starter cultures by experimental animals led to reduction of serum cholesterol concentration compared with control. The serum atherogenic index of animals that consumed raw smoked sausage with starter cultures from the MSUFP collection was the lowest. At the same time, the atherogenic index in experimental groups that consumed SKXX and BSK sausages tended to increase, while there was a decrease of atherogenic index in serum of animals that consumed SKK, although the differences were not statistically different in comparison with the control. Therefore, there is an obvious need for further research in this area.

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