Antibiotic resistant of microorganisms in fermented sausages

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Abstract. Fermented sausages, although considered safe to eat, sometimes cause serious bacterial infections in people, which can be fatal. We suppose that residual amounts of antimicrobial agents, including antibiotics in meat raw materials, can disrupt the fermentation process, contributing to the development of antibiotic-resistant strains of pathogens. Residual antibiotic amounts in meat have been studied. At least 30% of the samples contained antibiotics. Microorganisms were isolated from meat and minced meat used to produce fermented sausages. It was found that these microorganisms are resistant to antimicrobials. Salmonella showed the highest resistance to amoxicillin - 44.5%, gentamicin 38%; streptomycin 44.5% and tetracycline 55.5%. Bacteria of the species L. monocytogenes showed resistance to amoxicillin - 38.5%, benzylpenicillin - 30.8%, tetracycline 53.8% and ciprofloxacin 38.5%. Escherichia coli showed the highest resistance to amoxicillin - 33.3%, neomycin - 30%, streptomycin - 40% and tetracycline - 40%.

1. Introduction

Intensification of livestock farming has led to the extensive use of veterinary drugs, and it is estimated that 80% of all food-producing animals receive medication at least once in their lifetime [1, 2]. Fermented sausages are commonly considered safe for consumption, and the acidification by lactic acid starter bacteria is one of the main preserving factors. Nevertheless, outbreaks of serious sausage-borne gastrointestinal infections with pathogens such as verocytotoxific (shigatoxific) Escherichia coli (VTEC/STEC), Salmonella, and Listeria monocytogenes do occur regularly [2].

Although fermented beef products have been identified as potential health hazards [3], it is largely unclear when and why fermented sausages pose a risk to consumers. In sausages fermentation, starter cultures producing lactic acid are usually used to control the fermentation process in order to prevent the development of pathogenic microorganisms. At low antibiotic concentrations and acceptable levels, it was found that lactic acid bacteria are more susceptible to antibiotics than pathogenic microorganisms [4]. Minimum allowable concentrations of antibiotics in meat can inhibit the development of lactic acid microorganisms, but these concentrations do not affect the survival or even reproduction of pathogenic microorganisms.

The implication is that exposure to low levels of antibiotics should be prevented as much as possible, because this causes resistance far more than high concentrations that inhibit growth or kill the cell and thus prevent acquisition of resistance [5].
2. Materials and methods

2.1. Samples
The objects used were meat (pork, beef, poultry) and minced meat for fermented sausages obtained from a meat processing enterprise in the central region of Russia. Samples for this study were collected over a one-year period, from August 2018 to August 2019.

2.2. Microbiological methods for determining the presence of antibiotics
The ground test sample (25.0 ± 0.5 g) was homogenized with 25 cm³ of saline. Then, the container with the initial suspension was kept in a thermostat at a temperature of (37 ± 1) °C for 90 min periodically moving. After incubation, part of the initial suspension was placed in centrifugal tubes and centrifuged at 3000 rpm for 10 min. The presence of antimicrobial residues was determined as follows: the supernatant in an amount of 0.05 cm³ was transferred in parallel to two wells with Kundrat agar. Petri dishes with the test material were kept at room temperature for at least 30 min for diffusion of the supernatant fluid into agar; then they were placed in a thermostat (65 ± 1) °C for (3.5 ± 0.5) h. The lack of growth of the test culture Geobacillus stearothermophilus and the preservation of blue agar in a zone 2.0 mm wide or more was considered the presence of antibiotics in the test sample. The lack of growth of the test culture and the preservation of the blue color of the agar in the zone with a width of less than 2.0 mm or the presence of growth with a color change of the medium to yellow was considered the absence of antibiotics. Antimicrobial resistance was studied with broth macrodilution method. To detect the growth of microorganisms, the inoculated tubes were examined using transmitted light. The growth of a culture in the presence of antimicrobials was compared with the reference tube (a negative control) contained the initial inoculum.

2.3. Microbiological analysis
After the enrichment step, 0.1 cm³ aliquots were distributed on the surface of the culture media. To detect Salmonella spp. on Brilliance Salmonella agar (PCA, Merck) and incubated at 37 °C for 24 hours. To detect Staphylococcus aureus, seeded on Baird-parker agar (Merck) and incubated at 37 °C for 48 hours. To detect Listeria monocytogenes used the Chromocult Listeria Agar Ottaviani and Agosti (Merck) and incubated at 37 °C for 48 hours. To detect Escherichia coli chromogenic coliform agar (Merck) was used and incubated at 37 °C for 24 h. Microorganisms were identified using the MALDI Biotyper complex based on a Microflex tabletop time-of-flight mass spectrometer with a matrix laser desorption/ionization (MALDI-TOF) (Bruker Daltonik, GmbH).

2.4. Procedure for testing antibiotic sensitivity
To determine the susceptibility of microorganisms to antibiotics, antibiotic discs were used in various concentrations: ciprofloxacin (5 μl), streptomycin (300 μl), gentamicin (10 μl), amoxicillin (20 μl), neomycin (30 μl), benzylpenicillin (10ED) and tetracycline (30 μl). The inoculum of microorganisms was evenly distributed over the surface of the Mueller-Hinton agar. Then, antibiotic discs were placed on top and the plates were incubated at 37°C. After 24 h, the zone of inhibition of growth of microorganisms was taken into account. The diameters of the braking zones were recorded to the nearest millimeter and classified as susceptible, intermediate and stable according to [6].

3. Result and discussion

3.1. Results of monitoring meat for the presence of antimicrobial chemotherapeutic substances
The analysis of meat and minced meat used for the production of fermented sausages showed that at least 30% of the samples were contaminated with antimicrobial substances (figure 1).
Thus, the uncontrolled use of antibiotics in animal husbandry has led to the presence of antibiotic residues in animal slaughter products. Low concentrations of these antibiotics in the environment can cause random and spontaneous mutagenesis [7]. Therefore, the environment can be considered as a likely reservoir of antibiotic-resistant bacteria, as well as their resistance genes [8]. This situation is of serious concern to public health institutions around the world, since bacteria have the ability to transmit resistance genes between strains of the same species and between different species [9]. This is possible due to the fact that antibiotic resistance genes are located on elements, including transposons, integrons and plasmids, which can be immobilized.

To study the profile of antibiotic-resistant pathogenic and conditionally pathogenic microorganisms, meat and minced meat were monitored (figure 2).

The samples of raw meat and minced meat included in this study showed a high level of viable bacteria (figure 2). Most of the samples were contaminated with E. coli (23.5%). Other pathogenic
isolates were Listeria monocytogenes (20 %), as well as Salmonella spp. (14 %). Thus, E. coli, Salmonella spp. and Listeria monocytogenes are meat-borne public health pathogens.

3.2. Antibiotic susceptibility testing
Microorganisms isolated from meat and mince used for raw fermented sausages production have resistance to antimicrobial preparations. Bacteria of the genus Salmonella showed the greatest resistance to amoxicillin - 44.5%; Gentamycin 38; Streptomycin 44.5% and tetracycline 55.5%.

A study conducted by Alafi et al. also reported a high resistance to ampicillin, streptomycin, amoxicillin-clavulanic acid, cephalothin, cefotiofur, and cefoxitin [10]. Antibiotic resistance in foodborne pathogens such as Salmonella is a major concern for public health safety. In another study, Salmonella serovars such as S. typhimurium, S. lille, S. montevideo, S. hadar, S. meleagridis, S. cerro, S. kentucky, or S. muenster were identified from ground beef samples collected from 404 retail stores. Among these, five S. typhimurium isolates were resistant to ampicillin, streptomycin, sulfamethoxazole, ticarcillin, and tetracycline [11].

Bacteria of the species L. monocytogenes showed resistance to amoxicillin - 38.5%, benzylpenicillin - 30.8%, tetracycline 53.8% and ciprofloxacin 38.5%. In a similar study Yan et al. observed that 36.7% of L. monocytogenes isolates from different foods displayed resistance to one or more antibiotics and 18.9% of the isolates were multidrug resistant. Overall, antibiotic resistance was noticed in 14 of the 18 tested microorganisms. Two isolates were found resistant to more than five antibiotics [12]. In a similar study the resistance of Salmonella spp. to amoxicillin (2 strains), ciprofloxacin (1 strain), tetracycline (2 strains) and erythromycin (2 strains) was determined. Moreover, one of the strains is resistant immediately to the action of 3 antibiotics. The bacteria L. monocytogenes showed resistance to tetracycline (3 strains) and ciprofloxacin (1 strain). Both salmonella (1 strain) and L. monocytogenes (1 strain) strains with multiple antibiotic resistance were identified [13].

Escherichia coli exhibited the greatest resistance to amoxicillin 33.3%, neomycin 30%, streptomycin 40% and tetracycline 40%. Van et al. reiterated that food contaminated with antibiotic-resistant bacteria could cause amplification of resistance genes and facilitate the transfer of the antibiotic resistance determinants to other bacteria of clinical importance found in humans, and can be further transferred within humans to more pathogenic bacteria [14]. Therefore, food or animal-derived products, including meat, milk, and eggs, may represent an active and key medium through which antibiotic resistance determinants are continually being transferred between bacteria, and from animals to humans [15]. Bosco et al. clearly documented the multidrug resistance of Salmonella isolates recovered from cattle, pigs, chickens, eggs, and animal-derived products, as well as cross-species transmission of plasmids between animal and humans in Uganda [16].

4. Conclusion
The results of our studies demonstrate the presence of antibiotic residues in meat used for the production of fermented sausages. High levels of residues in some meat samples are troubling.

Resistant strains of salmonella, Listeria monocytogenes, and Escherichia coli are common in raw meat and minced meat used to make fermented sausages. The resistance of starter cultures to veterinary drugs has been investigated previously. Studies have shown that starter cultures can be exposed to even the smallest allowable amounts of antibiotics. This indicates food safety risks [17]. We concluded that fermented sausages made from meat with residual amounts of antibiotics, even at EU regulated levels, can cause serious foodborne infections.

These results confirm the need for guidelines for the wise use of antibiotics in food animals and for reducing the number of pathogens present on farms and slaughterhouses.

References
[1] Andersson D I and D Hughes 2012 Evolution of antibiotic resistance at non-lethal drug concentrations. Drug Resist. Update 15 162–72
[2] Moore J E 2004 Gastrointestinal outbreaks associated with fermented meats. Meat Sci. 67 565–8
[3] European Commission 2003. Opinion of the scientific committee on veterinary measures relating to public health on verotoxigenic E. coli (VTEC) in foodstuffs. http://ec.europa.eu/food/fs/sc/scv/out58_en.pdf
[4] Kjeldgaard J, Cohn M T, Casey P G, Hill C, Ingmer H 2012 Residual antibiotics disrupt meat fermentation and increase risk of infection. MBio 3 doi:10.1128/mBio.00190-12
[5] Van der Horst M A, Schuurmans J M, Smid M C, Koenders B B, Kuile B H 2011 De novo acquisition of resistance to three antibiotics by Escherichia coli. Microb Drug Resist 17 141-7
[6] CLSI Performance Standards for Antimicrobial Susceptibility Testing. 29th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2019
[7] Cogliani C., Goosens H., Greko C. Restricting antimicrobial use in food animals: Lessons from Europe. Microbe. 2011 6 274–9
[8] Marti R, Scott A, Tien Y-C, Murray R, Sabourin L, Zhang Y, Toppa E 2013 Impact of Manure Fertilization on the Abundance of Antibiotic Resistant Bacteria and Frequency of Detection of Antibiotic Resistance Genes in Soil and on Vegetables at Harvest. Appl. Environ. Microbiol 79 5701–9
[9] Chang Q, Wang W, Regev-Yochay G, Lipsitch M, Hanage W P 2015 Antibiotics in agriculture and the risk to human health: How worried should we be? Evol. Appl 8 240–5
[10] Alali W Q, Thakur S, Berghaus R D, Martin M P, Gebreyes W A 2010 Prevalence and distribution of Salmonella in organic and conventional broiler poultry farms Foodborne Pathog. Dis 7 1363–71
[11] Zhao T, Doyle M P, Fedorka-Cray P J, Zhao P, Ladely S 2002 Occurrence of Salmonella enterica serotype Typhimurium DT104A in retail ground beef J. Food Prot 65 403–7
[12] Abdollahazadeh E, Ojagh S M, Hosseini H, Ghaemii E A, Irajian G, Naghizadeh Heidarloo M 2016 Antimicrobial resistance of Listeria monocytogenes isolated from seafood and humans in Iran Microb Pathog 70-4
[13] Zaiko E V, Panchenko A A, satabaeva D M, Bataeva D S 2019 Prevalence and resistance to antibiotics of pathogenic microorganisms isolated from the meat of various animal species Vsyo o myase 3 42-6
[14] Van T.T.H., Moutafis G, Tran L T, Coloe P J 2007 Antibiotic Resistance in Food-Borne Bacterial Contaminants in Vietnam Appl. Environ. Microbiol 73 7906–11
[15] Wegener H C 2012 Antibiotic Resistance: Linking Humans and Animal Health IMPROVING Food Safety through a One Health Approach: Workshop Summary. Institute of Medicine, National Academic Press; Washington, DC, USA
[16] Bosco K J, Kaddu-Mulindwa D H, Asiiimwe B B 2012 Antimicrobial Drug Resistance and Plasmid Profiles of Salmonella Isolates from Humans and Foods of Animal Origin in Uganda Adv. Infect. Dis 2 151–5
[17] Bataeva D S, Minaev M Yu and Zaiko EV 2017 Effect of antibiotics on starter culture development in production of uncooked fermented sausages Meat industry 6 46-7

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