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Characteristics of Enterococci Isolated from Raw Milk and Hand-Made Cottage Cheese in Ukraine.

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ABSTRACT

An important and significant group of microorganisms of raw milk and dairy products compose bacteria of genus Enterococcus. These microorganisms are always present in milk and form the so-called primary micro flora. A serious problem remains the spread in food products of populations of enterococci with multiple antibiotic resistances. The article presents the results of study of species composition and antibiotic resistance of enterococci of raw milk and hand-made cottage cheese in Ukraine. It has been established that in cottage cheese among enterococci dominates E. faecalis (73, 4±6, 71%), its amount in 1, 37 times (p≤0, 05) more than in raw milk. Number of E. faecium bacteria in cottage cheese was 12, 1±1, 08%, which is 2, 9 times (p≤0, 05) less than in milk, and E. durians species was 5, 3±0, 47. It was found that 8, 1% of samples of raw milk was contaminated with VRE, while in cottage cheese was found 1, 7 times more of these bacteria. Mainly VRE of raw milk is represented by E. faecalis– 91, 6%, and in cottage cheese its amount does not exceed 7, 4%. Sensitivity of E. faecalis from cottage cheese to other antibacterial agents, which were taken in the study, was 1, 2 – 2, 5 times (p≤0, 05) less, compared with E. faecalis from raw milk.

Keywords: enterococci, raw milk, cottage cheese, antibiotic resistance.

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INTRODUCTION

An important and significant group of microorganisms of raw milk and dairy products are bacteria of genus Enterococcus [1, 6, and 7]. The main source of enterococci, where they are permanent residents and constitute a resident micro flora, is the intestine of human and animal [10, 13]. From this source they spread and pollute the environment. Therefore, researchers [4, 12] often reveal these bacteria in milk and dairy products, where they form the primary micro flora. Enterococci are used as probiotics and they are part of biologically active substances [18]. They as natural intestinal inhabitants take an active part in metabolic processes, synthesis of vitamins, hydrolysis of sugars, in particular lactose, deconjugation of bile acids, elimination of pathogenic bacteria. The quantitative content of enterococci in the intestine correlates with the level of content of other bacteria; in particular coli form bacteria, lactobacilli and bifid bacteria [14, 16]. Enterococci are effective immunostimulants, capable of maintaining adequate level of cytokines of wide spectrum for the normal functioning of congenital immune system [11]. Although most enterococcal species are not pathogenic to consumers, however, there are species that may cause food toxic infection [2, 3].

An important characteristic of enterococci, which causes increased interest, is the high natural resistance to many known antimicrobial agents [2, 3, and 4]. These bacteria exhibit increased resistance to antibiotics such as penicillins, amino glycosides, glycopeptides and other agents. An important problem remains the spread in food products of populations of enterococci with multiple antibiotic resistance and different sets of virulence factors that are of danger to humans [5, 8, and 9]. The spread of such strains scientists explain as the high resistance of enterococci to environmental factors, intensive and uncontrolled use of antibiotics and ability of enterococci to actively exchange genetic information, both between different species of genus and with heterogonous microorganisms [12, 15].

Also important from a scientific and practical point of view is the issue of spreading through food of vancomycin resistant enterococci (VRE) [4, 8, and 15]. This antibiotic belongs to reserve group and is used to treat staphylococcal, enterococci and other infections. Infections, caused by VRE, are characterized by rapid spread and relatively high level of morbidity and mortality among infected patients. One of causes of high mortality – significant constraints associated with the selection of effective etiotropic treatments for controlling these pathogens [17, 19]. Animal food products can be a VRE reservoir and play an essential role in spreading and transmitting them to humans. Therefore, the study of distribution of bacteria of the genus Enterococcus in foods, their species composition and sensitivity to antibacterial agents is relevant.

Purpose of work is to determine the specific composition and antibiotic resistance of enterococci of raw milk and hand-made cottage cheese in Ukraine.

MATERIALS AND METHODS

342 isolates of Enterococcus spp., separated over the past two years, were investigated in the study of 148 samples of raw milk and 194 samples of cottage cheese, selected on agricultural markets of Ukraine.

Isolation of enterococci was performed on Bile EsculinAzide Agar (Himedia, India). The crops were cultivated at temperature 37°C for 24-48 h.

Specific identification of bacteria was performed using the Enterococcus-test system (PLIVA-LachemaDiagnostika, Czech Republic) and the MALDI-TOF Biotyper software and hardware complex (BrukerDaltonics GmbH, Germany).

Chromogenic medium chromID VRE (BioMérieux, France) was used to isolate vancomycin resistant enterococci. To confirm VRE, the minimum inhibitory concentration of vancomycin and thiocoplane was determined by Etest method (Biomérieux, France) according to CLSI standards: vancomycin ≤4 μg/ml (sensitive), 8-16 μg/ml (low sensitivity), ≥32 μg/ml (resistant); and teicoplanin ≤8μg/ml (sensitive), 8-16 μg / ml (low sensitivity), ≥32 μg/ml (resistant).

Sensitivity of isolates to antibacterial agents (ampicillin (10 mcg), amoxyclav (30 mcg), ceftriaxone (30 mcg), ceftazidime (30 mcg), gentamicin (30mcg), kanamycin (30 mcg), lincomycin (10 mcg), tetracycline (30 mcg), norfloxacin (10 mcg), norfloxacin (5 mcg), levofloxacin (5 mcg), and nitrofurantoin (300 mcg)) were

determined by disc-diffusion method using antibiotic disks (Himedia, India). Mueller Hinton Agar (Himedia, India) was used when setting the method.

Statistical processing of results was carried out using methods of variation statistics using the program Statistical 7.0 (Stat Soft Inc., USA). Nonparametric methods of research were used (Wilcox on criteria, Mann-Whitney criteria). Arithmetic mean (x) and standard error of average (SE) were determined. The difference between comparable values was considered to be valid for P <0, 05.

RESULTS AND DISCUSSION

Specific identification was carried out and sensitivity to antibacterial agents of bacteria of the genus Enterococcus was determined. At the same time, the studies were planned to determine the specific composition of enterococci of raw milk and cottage cheese made from it, and then compare their resistance to antibacterial agents. The studies, conducted in this way, will determine the influence of manufacturing technology, namely the temperature on the species composition of enterococci and compare their sensitivity. Indeed, according to results [18], it is considered that the action of increased temperatures stimulates stress-proteins in enterococcal cells, which results in increased resistance to environmental factors: increased concentration of salt, pH, hydrogen peroxide, ethanol and others.

Results of identification of bacteria of the genus Enterococcus, which are separated from raw milk and cottage cheese, are given in Table 1.

Table 1: Species composition of bacteria of the genus Enterococcus, separated from raw milk and cottage cheese, entering the agricultural markets of Ukraine, %

Species of enterococci	Object of studies	
	Raw milk,n=148	Cottage cheese,n=194
E. faecalis	53,4±4,22	73,4±6,71
E. faecium	34,7±2,15	12,1±1,08
E. durans	9,3±0,74	5,3±0,47
Not identified species	2,6±0,17	9,2±0,82

n – Number of investigated samples.

As can be seen from Table 1, from raw milk we separated and identified three types of bacteria of the genus Enterococcus: E. faecalis, E. faecium and E. durians. The bulk of separated enterococci of milk is represented by the species E. faecalis – 53,4±4,22%, the proportion of E. faecium is 1,5 times less (p≤0,05) and was 34,7±2,15%, and the number of E. durians did not exceed 10% of all identified enterococci. Ereerococci, which exhibit related properties and are poorly differentiated, were 2, 6±0, 17%.

Domination in species composition of cottage cheese were E. faecalis microorganisms, which numbered 73,4±6,71%, which is 1,37 times (p≤0,05) more, compared to raw milk. The number of E. faecium bacteria in cottage cheese was 12, 1±1, 08%, which is 2, 86 times (p≤0, 01) less than their content in milk, and the species E. durians was 5, 3±0, 47%. Also was noticed increase 3, 5 times (p≤0, 01) of the proportion of unidentified enterococci species in cottage cheese, which was up to 9, 2±0, 82%, compared with milk.

Consequently, the conducted studies indicate that among the species composition of enterococci of raw milk and cottage cheese dominates the species E. faecalis, which is 53, 4±4, 22 and 73, 4±6, 71% respectively, and in our opinion it has fecal origin. Increase of 1,4 times of E. faecalis in cottage cheese is associated with additional contamination of it during the technology of manufacturing, storage and sale, or this species is more stable, compared to other species, to the temperature that is used during the process of cheese production. Excessive growth of this species is not only an indicator of violation of sanitary and hygienic requirements of production, but it can also be the cause of human infection with the product. This is due to the fact that the views on the presence of enterococci in foods are ambiguous. Some scientists consider them to be representatives of normal intestinal micro flora of humans, where they exhibit probiotic properties [1, 11]; while the other part tends to believe that this species is related to opportunistic pathogenic bacteria and can cause various inflammatory diseases, food poisoning in humans and animals [13].

Vancomycin resistant enterococci are of particular importance. Given the continuing presence of enterococci in intestines of animals, as well as the intensive use of antimicrobial agents for the prevention and treatment of bacterial infections, there is a real danger of human transmission of antibiotic resistance and virulence genes. However, despite the importance and practical significance of the problem of the spread of antibiotic resistant microorganisms in Ukraine, we do not have enough objective data on the potential reservoir and sources of gene distribution of antibiotic resistance of known human pathogens, including the vancomycin resistance genes.

Conducted studies show that 8, 1% of samples of raw milk were contaminated with vancomycin resistant enterococci, while at the same time, 1, 7 times more of these bacteria were detected from cottage cheese (Table 2). Identification of VRE revealed the presence of two species: E. faecalisand E. faecium. Mainly VRE of raw milk is represented by the species E. faecalis, since it accounts 91, 6%. In cottage cheese was noted an increase in the proportion of VRE due to E. faecium 2, 6 times, as compared with raw milk.

Table 2: Frequency of separation of vancomycin resistant enterococci from raw milk and cottage cheese, n = 342

Object of studies	Investigated samples		Number of VRE		Distribution of isolates					
					E. faecal is		E. francium		E. durians	
	n	%	n	%	N	%	n	%	n	%
Raw milk	148	100	12	8,1	11	91,6	1	8,4	0	0
Cottage cheese	194	100	27	13,9	20	74,1	7	22,2	0	0

The next part of this study was to determine and compare the sensitivity of E. faecalis to antibacterial agents used in human and veterinary medicine as the main representative of the genus Enterococcus, separated from raw milk and cottage cheese. Scientific studies point to the link between resistance to antimicrobial agents in bacteria that are separated from food and present in human microbiocenosis, with food being thought to be one of the ways of transmitting of antibiotic resistance [9, 15].

The results of studies of sensitivity to antibacterial agents of E. faecalis, which are isolated from raw milk and cottage cheese, are given in Table 3

Table 3: Antibiotic resistance E. faecal isisolated from raw milk and cottage cheese**, n = 104, %**

Type of antibiotic	E. faecal isisolated from raw milk, n=48		E. faecal isisolated from cottage cheese, n=56	
	n	%	n	%
Ampicillin, 10mcg	13	27,1	34	60,7
Amoxyclav, 30 mcg	1	2,1	8	14,3
Ceftriaxone, 30 mcg	8	16,7	23	41,1
Ceftazidime, 30 mcg	14	29,2	29	51,8
Gentamicin, 30 mcg	31	64,6	48	85,7
Kanamycin, 30 mcg	39	81,2	53	94,6
Lincomycin, 15 mcg	30	62,5	41	73,2
Tetracycline, 30 mcg	37	77,1	50	89,3
Norfloxacin, 10 mcg	25	52,1	36	64,3
Ofloxacin, 5 mcg	17	35,4	24	42,8
Levofloxacin, 5 mcg	23	47,9	31	55,3
Nitrofurantoin, 300 mcg	2	4,2	4	7,1

As can be seen from Table 3, E. faecalis microorganisms, separated from raw milk, in almost 100% of cases were sensitive to Nitrofuran, their resistance was 4,2%.

Of the three agents of fluoroquinolone series, the most effective against E.fecalis microorganisms was Ofloxacin, which inhibited growth in 64,6% of strains of cultures, and the sensitivity of Levofloxacin and Norfloxacin was 52,1% and 47,9% respectively.

Weak anti-enterococcal activity was shown by agents such as Tetracycline, Lincomycin, Kanamycin, Gentamicin, and the level of sensitivity to these antibiotics was from 18,8 to 37,5%. Cephalosporins of the third generation, Ceftazidime and Ceftriaxone, showed an average efficacy against the inhibition of E. faecalis bacteria from 70,8 to 83,3%.

E. faecalis showed significant sensitivity to β-lactam antibiotics: Amoxicillin and Ampicillin. At the same time, the resistance to Ampicillin was 27,1%, and to Amoxicillin with clavulanic acid was 2,1%. Obviously, clavulanic acid enhances anti-enterococcal effect in Amoxicillin.

It was established that the antibiotic resistance of E. faecalis bacteria, which are isolated from cottage cheese, is significantly higher than that of E. faecalis strains, which are isolated from raw milk. Thus, antimicrobial agents (Nitrofurantoin, Amoxicillin), which were almost 100% active to E. faecalis, separated from milk, showed lower efficacy to E. faecalis, separated from cottage cheese, the sensitivity was from 85,7 to 92,9%.

The sensitivity of E. faecalis from cottage cheese to other antibacterial agents that were taken in the study was 1,2 – 2,5 times ($p \leq 0,05$) less, compared with E. faecalis from raw milk.

Thus, summing up the conducted study, it can be noted that enterococci, separated from cottage cheese entering the agricultural markets, show increased resistance to antimicrobial agents, compared with enterococci, separated from raw milk.

These studies suggest that high milk acidity and prolonged temperature processing during the manufacture of cottage cheese acts as a stress that stimulates stress protein in E. faecalis cells and results in increased resistance to antimicrobial agents. Conducted studies show the detection of stress proteins responsible for the resistance of enterococci to environmental factors [11]. In addition, we believe in the possibility of breeding in cottage cheese of bacteria of the species E. faecalis with resistant properties to antibiotics. Entering the human gastrointestinal tract, E. faecalis can pass genes of resistance to pathogenic and opportunistic microorganisms. This is reported by studies [18] that indicate the possibility of transferring resistance genes from enterococci to tetracycline and erythromycin to listeria and lactic acid bacteria in the gastrointestinal tract of mice, and in vitro.

Thus, the studies suggest the possibility of breeding in cottage cheese, which is supplied for sale on agricultural markets, bacteria of the species E. faecalis with resistant properties to antibiotics. This, in turn, can form resistant strains of these bacteria in the gastrointestinal tract of humans – consumers of cottage cheese.

CONCLUSION

In cottage cheese in Ukraine among enterococci dominates E. faecalis, which is 73, 4±6, 71% that is 1, 37 times ($p \leq 0, 05$) more than in raw milk. The number of E. faecium bacteria in cottage cheese was 12, 1±1, 08%, which is 2, 9 times ($p \leq 0, 05$) less than in milk, and the E. durians species was 5, 3±0, 47.

It was found that 8, 1% of samples of raw milk were contaminated with VRE, while in cottage cheese 1; 7 times more of these bacteria were detected. Mainly VRE of raw milk is represented by E. faecalis– 91, 6%, and in cottage cheese its amount does not exceed 7, 4%. Sensitivity of E. faecalis from cottage cheese to other antibacterial agents, which were taken in the study, was 1, 2 – 2, 5 times ($p \leq 0, 05$) less, compared with E. faecalis from raw milk.

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