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DIHYDROQUERCETIN, THE BIOACTIVE SUBSTANCE, TO BE USED AGAINST PATHOGENIC MICROORGANISMS AS AN ALTERNATIVE TO ANTIBIOTICS

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Abstract

According to WHO reports, since 2000 a microorganism resistance to antimicrobials has become a serious threat to global public health. Thereby, more strict control for used antibiotics and novel antibacterial substances are considered helpful. Of that, the development of new antimicrobial compounds seems to be most perspective, seeing the high yielding animals are much more susceptible to diseases, and animal products are poorly stored because of microbial contamination. Among compounds possessing antimicrobial properties the dihydroquercetin, a bioflavonoid is of special interest due to wide range of biological activity, including antioxidant activity. Dihydroquercetin is used widely in food industry and medicine, but in animal farming its use is a novel project aimed to provide for animal welfare and quality of livestock products. We compared in vitro antimicrobial effect of different antibiotics (tetracycline, chloramphenicol, streptomycin, bacitracin, grisin, benzyl penicillin at 3.0, 5.0, 10.0, 16.0, 19.0, 24.0 and 48.0 μg/ml each) and 0.5, 1.0, 2,0 and 5.0 % dihydroquercetin to pathogenic, opportunistic, and probiotic microorganism *Staphylococcus epidermidis* ATCC 14990, *Micrococcus luteus* (*lysodeicticus*) ATCC 4698, *M. luteus* ATCC 10240, *Escherichia coli* VL-613, *Pseudomonas aeruginosa* 98. In gel diffusion test with a series of dilutions the diameters of growth inhibition zone (D) were measured. *St. epidermidis* was found to be high sensitive to 5.0 % dihydroquercetin (D of 21.33±0.82 mm) but low sensitive to all the tested concentrations of bacitracin at the Ds ranged from 14.40±0.27 to 18.80±0.42 mm, and to grisin at 3 to 10 μg per milliliter concentration with zone diameters of 18.30±0.22 to 19.80±0.22 mm. Probiotic *E. coli* and nonpathogenic *M. luteus* (*lysodeicticus*) ATCC 4698 и *M. luteus* ATCC 10240 of the gastrointestinal microflora seem to be insensitive to 0.5 до 2.0 % dihydroquercetin (D of 12.20±0.84 to 19.75±0.73 mm) but high sensitive to all tested antimicrobial drugs (D of 20.20±0.22 to 54.80±0.22 mm).

Keywords: antibiotic resistance, antibiotic sensitivity, dihydroquercetin, *Staphylococcus epidermidis*, *Micrococcus luteus* (*lysodeicticus*), *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa*.

World Health Organization (WHO) notes that microbial resistance poses a growing threat in the last twenty years. This resistance develops due to injudicious antibiotic use in healthcare and animal husbandry [1, 2]. WHO report on April 30, 2014, presented data on microbial resistance to antimicrobial drugs in more than 114 European and African countries for the first time (http://www.who.int/drug-resistance/documents/surveillancereport/en/).

Antibiotics in feed are widely used to stimulate the growth of healthy farm animals and to prevent their diseases. However, the use of antimicrobial drugs in large animal populations may result in spread of bacteria resistant to antibacterial drugs and may cause drug-resistant infections [3-6]. The problem is even more acute for pedigree cattle.

The higher number of resistant bacterial strains, which are common infectious pathogens for humans and animals, has the greatest epidemiological importance. Humans may be infected through contaminated food, after direct contact with animals, or though environment. In Europe, use of glycopeptides (e.g.,

avoparcin) to stimulate animal growth resulted in the spread of vancomycin-resistant enterococci in symbiotic flora and meat of animals as well as in symbiotic flora of healthy people [1]. Fluoroquinolones (e.g., enrofloxacin) induced ciprofloxacin-resistant *Salmonella*, as well as *Campylobacter* and *E. coli* causing human and animal diseases, which are difficult to treat [7, 8]. In EU countries, resistance to erythromycin is unevenly distributed among *Campylobacter* strains isolated from poultry and pigs; it is probably explained by differences in use of antimicrobial products. *E. coli* strains resistant to β-lactam antibiotics due to β-lactamase activity are isolated both from human patients and from farm animals [8, 9]. European Center for Disease Prevention and Control (ECDC) warns that growth of bacterial resistance to antibiotics poses a threat to humankind survival [10]. Moreover, microbial contamination contributes to losses of animal produce during storage [11].

When developing medicines, prebiotics, and probiotics, special attention is paid to biologically active substances. These substances include dihydroquercetin, an active antioxidant, immunomodulator, natural acceptor of free radicals, liver-protective drug, and drug protective against ionizing radiation. It has anti-inflammatory and analgesic properties, promotes vasodilation, impedes the progression of atherosclerotic plaques, and decreases cholesterol synthesis [12-16]; it also eliminates heavy metals, including radionuclides from the body because of its high complex properties. Dihydroquercetin is used in medicine and food industry; however, the available literature does not mention its use in animal husbandry as an alternative to antibiotics.

In this publication, we present for the first time the information on dihydroquercetin ability to suppress the growth of facultative pathogens without negative effects on principal species of normal flora of animals. These effects may form the basis for innovative program of replacing the feed antibiotics with this biologically active substance of natural origin.

The aim of this research was to study susceptibility of obligate and facultative pathogenic microorganisms to some antibiotics and dihydroquercetin in vitro.

Technique. Experiments were held with museum collection strains of *Staphylococcus epidermidis* ATCC 14990, *Pseudomonas aeruginosa* 98 (Tarasevich State Research Institute of Standardization and Control of Medical and Biological Products), *Escherichia coli* VL-613 (All-Russian Collection of Industrial Microorganisms, State Research Institute of Genetics, Moscow), *Micrococcus luteus* (*lysodeicticus*) ATCC 4698 (Research Center for Expert Review of Medical Goods of the Ministry of Health of Russia, Moscow), *M. coccus luteus* ATCC 10240 (All-Russian State Center of Quality and Standardization of Feeds and Veterinary Medicines, Moscow). The purity of cultures was confirmed before use by morphological, cultivation, physiological, and biochemical parameters. To prepare the test microbial inoculum, we inoculated several tubes with slanted meat-peptone agar with microbial culture and kept them in thermostat for 24 hours at 37±1 °C. Test culture was washed with 5 ml of sterile 0.9 % NaCl. The suspension density was adjusted to 3.3 by McFarland standard (equivalent to 1×10^9 CFU/ml). Inoculum was used within 15 min after its preparation.

Antibiotic standards produced according to specifications, in-house standards, state Reference Standards (RS; manufactured by VGNKI, Moscow) and added to the industry list were used in this study. The ampoules of tetracycline, chloramphenicol, streptomycin, bacitracin, grisin, or benzylpenicillin were weighed and reconstituted with sterile 0.9 % NaCl. Working solutions of the following concentrations were made: 3.0, 5.0, 10.0, 16.0, 19.0, 24.0, and 48.0 μg/ml. To prepare dihydroquercetin working standard, 0.5 g of the product (Ametis, Russia)

was dissolved in 10 ml of sterile distilled water. Working solutions of the following concentrations were made: 0.5 %, 1.0 %, 2.0 %, and 5.0 %. The obtained cultures were steam sterilized (1 atm, 112 °C, 15 min) and cooled to room temperature.

The microbial susceptibility to antibiotics and dihydroquercetin was determined by diameter of growth inhibition due to diffusion of antimicrobial compounds into the agar [17]. Test culture was added (0.5 ml per 50 ml of the medium) into the melted growth medium for determining the microbial susceptibility to antibacterial drugs, prepared according to instructions (State Research Center of Applied Microbiology and Biotechnology, Moscow Province), and cooled to 40-45 °C; 20 ml aliquots were poured into Petri dishes. Agar was dried in a thermostat for 1-2 hours; then 5 holes were made using drill of 8 mm (±0.1 mm) outer diameter, 6 mm (±0.1 mm) inner diameter, and height of 10 mm (±0.1 mm).

Each sample of antibacterial drug and dihydroquercetin was tested in 4 dishes. To the first hole, 0.06 ml of 0.9 % NaCl was introduced; into other holes, 0.06 ml of antibiotic or dihydroquercetin solution in the concentration tested was added. Dishes were put into a thermostat for 24 hours at 37±1 °C. After incubation, they were placed bottom up on a dark matt surface, and diameters were measured in reflected light at incidence angle of 45°. Growth inhibition diameters were measured with accuracy up to 1 mm, using vernier caliper. Cultures were deemed resistant if diameter of growth inhibition was less than 15 mm, of intermediate susceptibility if diameter was 16-19 mm, and susceptible if diameter exceeded 20 mm [17, 18].

MS Excel software and parametric methods were used for statistical data processing. This paper presents thee arithmetic means and the mean errors.

Results: The following table shows the obtained data.

Growth inhibition diameters ($X \pm x$, mm) of microbial test cultures exposed to antibiotics and dihydroquercetin

Antimicrobial agent, concentration	*Escherichia coli* VL-613	*Micrococcus luteus* (*lysodeicticus*) ATCC 4698	*Micrococcus luteus* ATCC 10240	*Staphylococcus epidermidis* ATCC 14990	*Pseudomonas aeruginosa* 98
Dihydroquercetin (%)					
0.5	12.20±0.84	15.00±0.71	13.22±0.16	12.00±0.35	11.60±0.17
1.0	12.40±0.27	16.46±0.28	16.50±0.32	16.67±0.41	14.40±0.23
2.0	14.80±0.55	19.75±0.73	17.90±0.46	19.67±0.82	15.70±0.16
5.0	15.60±0.42	23.25±0.71	20.80±0.42	21.33±0.82	17.50±0.18
Benzylpenicillin (μg/ml)					
3.0	11.80±0.22	45.40±0.27	45.60±0.27	17.20±0.22	27.20±0.22
5.0	12.80±0.22	—	46.60±0.27	18.60±0.27	30.80±0.22
10.0	15.60±0.27	47.00±0.35	49.60±0.27	20.20±0.22	32.00±0.50
16.0	15.60±0.22	49.60±0.27	50.80±0.42	20.80±0.22	32.80±0.22
19.0	15.80±0.27	50.80±0.42	—	—	—
24.0	18.00±0.18	54.80±0.22	54.60±0.27	22.40±0.27	37.40±0.27
48.0	11.80±0.22	45.40±0.27	45.60±0.27	17.20±0.22	27.20±0.22
Grisin (μg/ml)					
3.0	15.00±0.18	21.40±0.27	24.20±0.22	18.30±0.22	14.60±0.27
5.0	15.20±0.22	23.20±0.22	24.80±0.22	18.60±0.27	15.80±0.42
10.0	15.80±0.22	—	26.00±0.35	19.80±0.22	18.00±0.61
16.0	16.00±0.00	23.60±0.27	26.40±0.27	20.00±0.00	18.60±0.27
19.0	16.60±0.27	26.20±0.22	—	20.60±0.27	—
24.0	—	27.67±0.41	28.00±0.18	—	19.80±0.22
48.0	18.20±0.22	29.67±0.41	30.00±0.35	23.60±0.27	21.80±0.22
Tetracycline (μg/ml)					
3.0	—	29.88±0.36	—	11.80±0.29	—
5.0	—	31.60±0.27	—	11.80±0.22	—
10.0	—	35.80±0.22	—	14.00±0.35	—
16.0	—	36.60±0.27	—	14.70±0.14	—
19.0	—	39.40±0.27	—	14.70±0.22	—
24.0	—	42.67±0.33	—	–	—
48.0	—	50.67±0.33	—	16.50±0.29	—
Streptomycin (μg/ml)					
3.0	18.80±0.22	23.33±0.22	26.00±0.32	24.40±0.27	15.80±0.42
5.0	19.40±0.27	—	34.60±0.27	26.00±0.50	17.00±0.50

Table continued

10.0	19.80±0.22	24.67±0.22	35.73±0.13	27.00±0.35	23.80±0.22
16.0	20.20±0.22	24.67±0.22	39.67±0.29	27.80±0.22	23.80±0.22
19.0	—	29.00±0.58	—	29.80±0.22	—
24.0	20.60±0.27	35.33±0.33	39.90±0.09	—	25.00±0.00
48.0	26.76±0.17	37.33±0.33	44.67±0.29	30.90±0.55	27.80±0.55
Bacitracin (μg/ml)					
3.0	26.67±0.37	38.75±0.29	39.67±0.37	14.40±0.27	12.90±0.23
5.0	27.60±0.27	40.67±0.41	42.67±0.41	15.80±0.22	15.40±0.21
10.0	28.60±0.27	—	44.00±0.35	17.40±0.27	17.00±0.18
16.0	—	41.67±0.41	—	—	—
19.0	30.60±0.27	—	45.67±0.41	18.80±0.42	18.40±0.45
24.0	—	42.67±0.41	—	—	—
48.0	—	—	—	—	—
Chloramphenicol (μg/ml)				—	
3.0	25.40±0.27	32.20±0.22	—	30.60±0.27	22.80±0.42
5.0	25.60±0.27	–	—	34.20±0.42	—
10.0	26.60±0.45	33.60±0.27	—	34.80±0.22	26.40±0.42
16.0	28.40±0.27	34.80±0.22	—	39.80±0.22	28.20±0.27
19.0	—	36.60±0.27	—	40.60±0.27	—
24.0	30.40±0.27	37.40±0.27	—	—	30.40±0.27
48.0	31.60±0.27	37.60±0.27	—	41.00±0.35	30.20±0.22

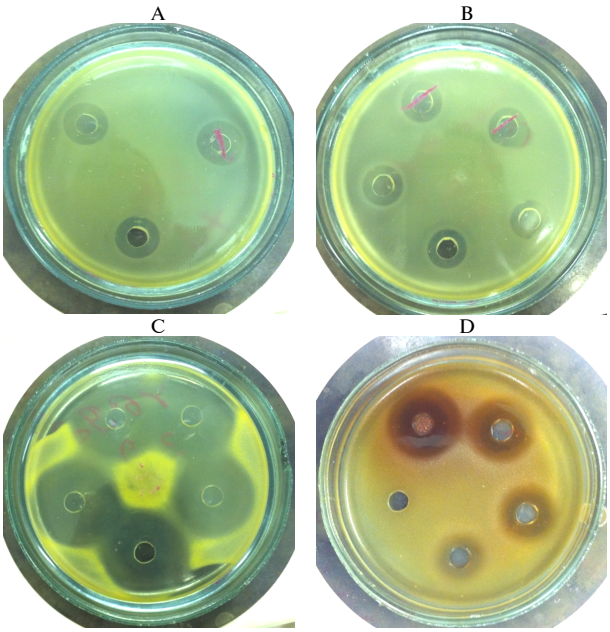
N o t e. Agar diffusion method was implemented. Cultures were deemed resistant if diameter of growth inhibition was less than 15 mm, of intermediate susceptibility if diameter was 16-19 mm, and susceptible if diameter exceeded 20 mm. Dashes mean that the respective parameter was not measured.

St. epidermidis are gram-positive cocci. It showed maximum susceptibility to all concentrations of chloramphenicol (growth inhibition diameter 30.60±0.27 mm to 41.00±0.35 mm) and streptomycin (24.40±0.27 mm to 30.90±0.55 mm) and to high concentrations (16-48 μg/ml) of grisin (20.00±0.00 mm to 23.60±0.27 mm) and benzylpenicillin (20.20±0.22 mm to 22.40±0.27 mm). The test culture demonstrated resistance to tetracycline (Fig. A) and bacitracin (3.0-48.0 μg/ml), to low levels (3.0-10.0 μg/ml) of grisin and benzylpenicillin. At the same time, *St. epidermidis* was poorly susceptible to 0.5 % and 1.0 % dihydroquercetin (growth inhibition diameter was 12.00±0.35 mm and 16.67±0.41 mm, respectively) (see Fig. B) but highly susceptible to 2.0 % (19.67±0.82 mm) and 5.0 % dihydroquercetin solutions (21.33±0.82 mm).

Ps. aeruginosa are gram-negative facultative pathogenic bacteria. The tested strain showed high susceptibility to chloramphenicol (growth inhibition diameter 22.80±0.42 mm to 30.20±0.22 mm), benzylpenicillin (27.20±0.22 mm to 37.40±0.27 mm), streptomycin (23.80±0.22 mm to 27.80±0.55 mm), grisin in concentration of 48 μg/ml (21.80±0.22 mm), and 2.0 % and 5.0 % dihydroquercetin (15.70±0.16 mm and 17.50±0.18 mm, respectively). However, the test culture was poorly susceptible to low concentrations of grisin and streptomycin and to all bacitracin concentrations.

E. coli is used in livestock farming as a probiotic culture [19, 20]. In our tests, growth inhibition diameter for *E. coli* VL-613 exposed to 0.5 % dihydroquercetin was 12.20±0.84 mm, the antimicrobial effect being lower than with the tested antibiotics in the applied concentrations; as for the use of 1.0 % dihydroquercetin, the parameter (12.40±0.27 mm) was similar to that after benzylpenicillin concentration of 5.0 μg/ml. The effect of 2.0 % dihydroquercetin solution (14.80±0.55 mm) was approximately similar to that observed with grisin in concentration of 3 μg/ml (15.00±0.18 mm) and benzylpenicillin in concentration of 10 μg/ml (15.60±0.27 mm). Growth inhibition diameters of 5.0 % dihydroquercetin solutions (15.60±0.42 mm) were approximately equal to those of streptomycin at concentration of 3 μg/ml (18.80±0.22 mm), grisin at 5 μg/ml (15.20±0.22 mm), and benzylpenicillin at 16 μg/ml (15.60±0.22 mm). *E. coli* was highly susceptible to all chloramphenicol and bacitracin concentrations; the minimum tested level (3 μg/ml) produced growth inhibition diameters of 25.40±0.27 mm and 26.67±0.37 mm, respectively, being significantly higher than

the respective parameter with the maximum concentration of dihydroquercetin.



The susceptibility of *Staphylococcus epidermidis* ATCC 14990 (upper row) **and *Micrococcus luteus* ATCC 4698 strains** (lower row) **to tetracycline** (A — 48 μg/ml; C — 3 μg/ml) **and dihydroquercetin** (B — 1.0 % solution, D — 0.5-5.0 % solution).

M. luteus are gram-positive immotile cocci. They play a minor role in human or animal diseases. Our experiments showed that the test strain of *M. luteus* (*lysodeicticus*) ATCC 4698 was highly susceptible even to minimum antibiotic concentrations. Thus, growth inhibition diameter for antibiotics (3.0 μg/ml) ranged from 21.40±0.27 mm to 45.40±0.27 mm (see Fig. C), while even the maximum concentrations of dihydroquercetin (2.0-5.0 %) were less inhibitory against this culture (growth inhibition diameter 19.75±0.73 mm to 23.25±0.71 mm) (see Fig. D). The activity of antibiotics was similar against *M. luteus* ATCC 10240. At 3.0 μg/ml, growth inhibition diameter ranged from 24.20±0.22 mm to 39.67±0.37 mm and 30.00±0.35 mm to 54.60±0.27 mm at the maximum level (48.0 μg/ml). At the same time, the culture was poorly susceptible to all tested concentrations of dihydroquercetin (growth inhibition diameter was 13.22±0.16 mm and 20.80±0.42 mm for 0.5 % and 5.0 % solutions, respectively).

Thus, facultative pathogens such as *Staphylococcus epidermidis* ATCC 14990 and *Pseudomonas aeruginosa* 98 demonstrated poor susceptibility to all bacitracin concentrations and to some grisin concentrations (3-16 μg/ml), while probiotic *Escherichia coli* VL-630 and nonpathogenic *Micrococcus luteus* (*lysodeicticus*) ATCC 4698 and *M. luteus* ATCC 10240 were highly susceptible to these drugs. These results cast doubt on feasibility of use of the above substances as antibiotics added to animal feed. However, dihydroquercetin solutions effectively suppressed facultative pathogens, being without negative effects on probiotic cultures. Thus, *St. epidermidis* and *Ps. aeruginosa* showed high susceptibility to 2.0 % and 5.0 % dihydroquercetin, while *E. coli* VL-630, *M. luteus* (*lysodeicticus*) ATCC 4698, and *M. luteus* ATCC 10240 showed low susceptibility. We think that dihydroquercetin may be suggested as alternative to feed antibiotics because this compound of biological origin is able to inhibit the growth and development of facultative pathogens without negative effects on key species present in normal flora of animals.

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