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Probiotics Based On L. Plantarum Vkpm B-2347 And Pr. Freudenreichii Vkpm B-6561 Strains And The Prospects For Their Preventive Use In Farming.

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ABSTRACT

Some of the criteria of microorganism inclusion in the group of probiotics are used to stabilize the intestinal flora, to be non-pathogenic, non-toxic, non-carcinogenic and non-invasive ones for a body. There are data on the use of lactose and propionic acid bacteria as probiotics. Invivo test of the composition from the strains Lactobacillusplantarum strain VKPM B-2347 and Propionibacteriumfreudenreichii strain VKPM B-6561 confirmed the drug safety, the lack of undesirable side effects. It was showed that the tested suspension makes an antagonistic effect of Staphylococcusssp., Salmonella, Shigella, Proteus, improves the growth of Lactobacillussp bacteria. The optimization level of rabbit gut microbiota rabbits depending on a dose may be extrapolated to other mammals.

Keywords: Microbiocenosis, probiotic, bacteria, Propionibacteriumfreudenreichii, Lactobacillusplantarum.

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INTRODUCTION

Probiotics, as one of the classification units of biological products retain its use validity for decades. According to some requirements of Food and Agriculture organization of the United Nations and WHO [1] probiotic microorganisms should be non-pathogenic and non-toxic ones, resistant to biological fluids of a gastrointestinal tract and to the long-term storage, have adhesion and antagonism properties to pathogenic and conditionally pathogenic microorganisms. A variety of production technologies and the components for microbial products develop the basis for interaction study between probiotic bacteria metabolites and macroorganism systems, in particular with intestinal biota. The microbial picture of animal and human intestine is represented mainly by anaerobes, the ratio of which in a norm provides the colonization resistance, an anti-toxic effect, the maintaining of metabolic and enzymatic process and immune status optimal level [2, 3, 4].

Propionic acid bifidobacteria, lactobacilli, streptococci, enterococci, the representatives of the E. coli, spore-forming bacilli and some yeast-like organisms are widely used as the means for disbacteriosis prevention in medical and veterinary practice, as well as in the food industry [5, 6, 7, 8].

Numerous studies of multicomponent microbial drugs proved the efficacy and advantages of their use. There are several complexes containing probiotic strains of Propionibacterium and Lactobacillus bacteria [9, 10, 11]. The combination of Pr. freudenreichii and L. plantarum as the autochthonous microorganisms of a gastrointestinal tract of a man and mammals [12, 13] is of interest in order to study their effects on an organism. Pr. freudenreichii produce formic, acetic, lactic, propionic acid, benzoic acid and free fatty acids, CO2, bacteriocines (propiocines), however, they have relatively low antibacterial activity [14, 15, 16].

Lactobacillus Plantarum synthesizes the antibiotic laktolin [17], bacteriocins, and also uses the mannose-specific adhesion places on the intestine wall as an opportunity to compete with other microorganisms for nutrient substrates [18, 19].

The World Health Organization in collaboration with the Food and Agriculture Organization of the United Nations developed clear guidelines for the evaluation of probiotic products. Thus, the probiotic properties of bacteria and their physiological benefit must be proven by the experiments on laboratory animals and confirmed by clinical trials [20, 21].

This work was carried out in order to determine the probiotic safety on the basis of Lactobacillusplantarum and Propionibacteriumfreudenreichii strains and the study of their impact on the dynamics of the intestine normal flora, depending on the amount of administered drug.

MATERIALS AND METHODS

The object of research was the microbiological preparation based on collection strains of bacteria from the Museum of the Russian National Collection of Industrial Microorganisms (VKPM) under the State Research Institute of Genetics. The strain IC-762-2-3 Lactobacillusplantarum is deposited at VKPM under the number B-2347, the strain IC-763-3-4 Propionibacteriumfreudenreichii is deposited under the number B-6165. The subject of research is the microflora of laboratory animal large intestine, 45 day old rabbits of Soviet Chinchilla species as a reference.

Table 1. The scheme of Lactobacillus and Propionibacterium microbial suspension application for Soviet Chinchilla rabbits

Control group	1 – experimental group	2 – experimental group
The drug was not administered	The drug with potable water 1 time per day at the dose of 0,25 ml/kg for 30 days	The drug with potable water 1 time per day at the dose of 0,5 ml/kg for 30 days

The analysis of experimental rabbit intestinal microflora was performed each 10 days during the period of the drug administration every 30 days after the cessation of drug provision. Caecotrophs were used for research. Experimental studies were conducted in LLC "NPF Research Center" of Koltsovo village.

The studied specimen of colon content were treated for the first 8 hours after the sampling. The microbiological studies of colon microbiocenosis are reduced to the identification and a qualitative accounting of bacteria number: Bifidobacteriumspp., Lactobacteriusspp., Enterobacteriumspp., E. Colispp., Str. haemolitycusspp., Salmonellaspp., Clostridiumspp., Staphylococcusspp., Candidaspp., Klebsiella spp. [22].

The samples after several subsequent dilutions (10-2 - 10-8) with sterile peptone solution with tween were plated on solid and liquid media. In order to determine the bacteria of the Enterobacteriaceae family the sowing on Endo Agar, Levin, Ploskirev media, blood agar (for simultaneous registration of hemolytic E. coli) and bismuth - sulphite agar was performed followed by the study of colonies. Then, the obtained colonies were sowed with streaks along the stock and the injection in a combined medium column for the primary identification (Olkenitsky media). The ability to ferment lactose (the representatives of Escherichia, Klebsiella, Citrobacter, Enterobacter species) was evaluated by the color of the combined medium oblique part.

In order to determine the bacteria of Bifidobacterium family the series of diluted samples with with tween peptone solution were sown in test tubes with liquid thioglycollate medium; the identification of Lactobacillus representatives was performed by sowing on Blikfeld media.

In order to determine the quantitative content of the Enterococcus bacteria the sowing on polymixine agar were performed. The identification of staphylococci (Staphylococcus family) was performed on vitelline-salt agar.

The accounting of results was performed by the means of colony counting and according to Baymuratova et al. method [23] using the following formula: $M = N \times 10n + 1$,

where M - the number of bacteria per gram of feces; N - the number of developed colonies on a plate; n - the dilution level of the material under study. The method of CFU/g calculation.

Microbiological studies were focused on the allocation of certain types of bacteria from mixed natural population, the cultivation on artificial nutrient media, ensuring the preservation of the basic biological microbe properties to determine the species specificity.

The digital materials were processed the statistical processing program SNEDECOR V4, PGN, Microsoft Excel. The reliability of the study results was determined according to the Student's test.

The materials of previously conducted research were used in the laboratory of biotechnological monitoring of LLC "NPF Research Center" at Koltsovo v. for the study of acute, subchronic, chronic toxicity of probiotic drug based on Propionibacteriumfreudenreichii and Lactobacillusplantarum, long-term effects of its use (teratogenicity, mutagenicity, carcinogenicity, immunotoxicity, allergenicity).

RESULTS AND THEIR DISCUSSION

The process of probiotic development based on Propionibacteriumfreudenreichii Lactobacillusplantarum includes classical stages: the breeding of strains - producers on selective nutrient media, the development of seeds, fermentation, preparation of the final formulation and packaging.

According to reports on the testing of the drug containing Lactobacillusplantarum VKPM B-2347, Propionibacteriumfreudenreichii VKPM B-6165, the animal models obtained the certificate of safety for this complex of strains. Thus, a series of experiments revealed that the drug during the admission through the stomach and the application on the skin of mice did not cause any toxic effects, a significant change in general condition, behavior, appetite, hair coat, mucous membranes, body weight, and death of animals, and it also did not show the ability to accumulation in the organisms of mammals.

The evaluation of the impact on the male and female gonads within the complex of probiotic lactobacilli and propionic acid bacteria was carried out on the basis of indirect indicators - as the result of embryonic and post-embryonic development of the offspring. The experiments showed no adverse effect of the feed additive on the male and female gonads. Such indicators as weight, young animal survival, as well as

the number of dead animals did not differ from control (intact ones) and experimental animals which received the drug.

The level of probiotics impact on the reproductive function was assessed on the basis of the offspring prenatal and postnatal development indicators. There were no statistically significant differences between control and experimental groups of animals on such indicators as the intrauterine death of embryos, the number of ugly embryos, the weight and craniocaudal size of embryos, the offspring survival and development dynamics.

Feeding a suspension containing lactobacilli and propionic acid bacteria to the animals during pregnancy did not result into embryo death and embryo ugly appearance, the delay or disruption of their development. The feeding of the drug did affect the postnatal development of the offspring. The dynamics of the young animal development and the survival rate in the test versions were not significantly different from control ones.

The experiments showed that the probiotic based on Propionibacteriumfreudenreichii and Lactobacillusplantarum does not induce the formation of revertants among three indicator strains of Salmonella. The addition of microsomal activating mixtures also did not result in a statistically significant increase of revertant number per plate. At that the number of revertants increased sharply in the positive control variants using nitrosomethylurea, 2 nitrofluorene and cyclophosphate. These compounds are increased the amount of formed revertants more than 100-fold, so they may be attributed to strong mutagens. In the control options using the nutrient medium the statistically significant increase in the number of revertants was not observed.

The experiments with the mutant strains of Escherichia coli auxotrophic by tryptophan showed that the addition of the feed additive suspension in the amount of (0.01-0.5) cm^3 per cup does not affect the growth rate of the test cultures. The delay of test culture growth was noted at the doses greater than 1 cm^3 per cup. This delay is probably related to the bactericide properties of the drug. On the basis of these experiments we may conclude that the preparation has no mutagenic properties against Escherichia coli strains.

Operation showed that probiotic on the basis of lactobacilli and propionic acid bacteria has no ability to induce mutations among indicative microorganisms. The experimental study of Lactobacillusplantarum and Propionibacteriumfreudenreichii mutagenic effect on mammalian organism stated that the administration of the drug in the stomach does not result in the induction of dominant lethal mutations (DLM) within the germ cells of male rats. Similar results were presented during the drug impact analysis on the body cells of rat bone marrow. Thus, on the basis of metaphase cell analysis of bone marrow the increase of cell number with chromosome and chromatid type disorders and after probiotic exposure was not stated. The number of cells with gaps among the experimental and control animals was also on the same level.

The work showed that the studied drug at intragastric and intraperitoneal administration results in a slight increase of the mass and cellularity of immunity organs. The cell viability of studied organs at intragastric introduction of the drug remained on the level of control. When a probiotic was administered through skin we did not register any significant changes in mass, cellularity and viability of cells of mouse immunity organs.

The introduction of propionibacteria and lactobacilli complex intraperitoneally and intramuscularly increases the number of rosette forming T-lymphocytes in thymus, and the phagocytic activity of mouse macrophages. On the contrary, when the drug was applied on skin we did not register any significant changes in the number of immune rosette forming cells of T-lymphocytes in thymus and the phagocytic activity of mouse macrophages.

During the evaluation of probiotic effect on the number of antibody producing B - lymphocytes (AOK) it was found that intraperitoneal injection of the drug suspension results in a significant increase of their number in the spleen. However, the differences showed statistical significance - at 0.5% level of significance. When the feed additive is applied on the skin surface the significant changes in the number of antibody producing B - lymphocytes (AOK) were not recorded.

The administration of the test drug to white mice and rats does not lead to a substantial increase in premature death of animals and a significant increase in the formation of internal organ tumors. On the contrary, under the effect of the feed additive the animals did not demonstrate the number of tumor decrease.

During the study of the allergenic effect of the feed additive on mongrel guinea pigs of laboratory population it was determined that it has weak allergenic properties and is characterized by 1 point according to 6-point scale.

The development of anaphylactic shock under the influence of the probiotic was analyzed. During the operation it was found that at intracardiac drug suspension administration the anaphylactic shock symptoms were recorded in 5-7 minutes. Intraperitoneal and subcutaneous injection of the drug caused weakening and protracted anaphylactic reaction. Overall, during the first minutes after the drug injection we registered the manifestation of intense movement of jaws and the carding of faces. The duration of the shock made 15-30 minutes, followed by the restoration of a body normal condition. In general, it was found that the drug may cause the anaphylactic shock of a mild severity.

In a series of experiments, the drug ability to cause an allergic reaction among guinea pigs was studied during its introduction through the skin. It was found that during the application of the drug on the skin for 10 and 20 days the animals do not demonstrate irritation reactions. The weak skin irritation, in the form of diffuse redness was reported among 30% of experimental animals after 2 months of application.

The research results developing with the age of rabbit gut microbiota demonstrate the similarity of indicators at oral administration of lactobacilli and propionibacteria in comparison with control samples. At the beginning of the test the analysis of the microbial landscape among the studied rabbits showed no statistically significant differences.

Significant differences are traced during the experiment at the statistical treatment of all study group data. Thus, the average number of colony forming units (CFU) of bifidobacteria per gram of rabbit biomaterial rabbits of the first and the second experimental group is increased on the 30th day of the experiment (and on the 75th day after birth) to 1010, which is 90% more than in the control group indicators ($p \leq 0.05$) and the amount is maintained at this level throughout the experiment.

During the ontogenesis of animals of 1st and 2nd groups the content of Lactobacillusssp. in the samples of caecotrophes is significantly higher at the comparison of these figures with the control ones (Table. 2) on the 20-th and the 30-th day (at the age of 65 and 75 days) by 43% (R≤0,01) and 43% (R≤0,01); 28% ($p \leq 0.05$) and 43% ($p \leq 0.05$), respectively. After the cessation of the suspension L.plantarum and Pr.freudenreichii introduction the average values of lactobacillus are also higher in the 1st and 2nd group at the 60th and 90th day of the experiment (at the age of 105 and 115 days) by 22% (R≤0 05) and 27% ($p \leq 0.05$); 10% ($p \leq 0.05$) and 10% ($p \leq 0.05$), respectively, compared to control counterparts (Table 3).

Assessing the statistically processed data of Enterococcusssp. we note the significant changes in CFU per gram of biological material under the impact of Veles preparation 6.59 within the dose of 0.25 mg/kg: the 10th day of the experiment (at the age of 55 days) the increase made 21% ($p \leq 0.05$), on 20th and 30th day (at the age of 65 and 75 days) the decrease made 16% ($p \leq 0.05$) and 15% ($p \leq 0.05$), respectively, compared with similar control groups. At the introduction of lactose and propionic acid microorganism doses within the dose of 0.5 mg/kg a significant decrease of enterococci indicators on the 20th and 30th day of the experiment is observed (at the age of 65 and 75 days) by 15% (R≤0,05) and 22% ($p \leq 0.05$), respectively, in comparison with rabbit indicators for which the probiotic was not fed (Table 2).

After the cessation of Veles drug administration 6.59 (Table 3) the quantitative values of Enterococcusssp. bacteria were significantly lower only in the 2nd test group by 20% ($p \leq 0.05$) at the age of 105 days (on the 60th day of the experiment) compared with the control results, whereas the animals of the 1st group have a slight decrease of these indicators.

In respect to lactose-negative microorganisms of the Enterobacteriaceae family according to the data of Table 2 and 3 the dependence of their amount is traced on the dose of the introduced drug. During the period from 10-th to 60-th day of the experiment (at the age of 55 - 105 day) we note that during and after the appointment of Veles 6.59 at the amount of 0.25 mg/kg the indicators of bacteria with reduced enzymatic activity was significantly lower by 10-21% ($p \leq 0.05$) in comparison to the analogs of the control group. Along with the obtained data the introduction of Veles 6.59 in the dose of 0.5 mg/kg significantly increased the number of lactose negative enterobacteria on the 20th day of the experiment (at the age of 65 days) by 16% ($p \leq 0.05$), followed by the reduction on the 30-th and the 60-th day (at the age of 75 and 105 day) - by 10% ($p \leq 0.05$) and 20% ($p \leq 0.05$), respectively, in comparison with indicators of the rabbits for which probiotic was not fed.

In the course of the performed surveys it was found that there were no significant differences of statistically processed data of CFU per gram of lactopositive bacteria Enterobacteriaceaessp. in the gut microbiocenosis of all test animals.

The average number of colony forming units Staphylococcusssp. is significantly less from 20-th to the 90-th day of study (at the age of 75, 135 days) in the first experimental group than the control group data in the range of 8-17% ($p \leq 0.05$). In the second group the same effect is observed only from the 60-th to the 90-th day of the experiment with the decrease by 7% ($p \leq 0.05$) and 17% ($p \leq 0.05$), respectively. At that a significant reduction of staphylococci is observed among the animals with a designated drug within the doses of 0.25 and 0.5 ml/kg which takes place at the end of the test.

The bactericidal action of the used allochthonous microorganism suspension is noted in haemolytic E.coli (Table 2). Thus, the decrease of pathogenic E. coli till the complete absence in samples was observed on the 20-th day of the experiment (at the age of 65 days) when rabbits were taken Veles 6.59. There were no significant differences of yeast counting among all tested animals.

It is known that L.plantarum is a direct antagonist and it inhibits S.aureus activity, and also causes the delay of indigenous Lactobacillussp. growth, but does not change their antagonistic properties [24, 25]. In its turn Pr. freudenreichiissp. shermanii has antibiotic activity and bactericidal action against Enterococcusfaecalis, Salmonellasp., Staphylococcusaureus, Escherichiacoli., Klebsiellapneumonia, Shigellasonnei [26, 27], thus providing a positive effect on the growth of lactic bacteria [28] and bifidobacteria [29]. The effects of probiotic microorganism action within the framework of our experience are consistent with the above stated data.

During the conducted studies it was established that the introduction of L.plantarum and Pr.freudenreichii probiotic strains symbiotic complex and within the dose of 0.25 ml/kg is caused by a marked increase of lactobacilli, the lowering of lactosonegative Enterobacteriaceae and Staphylococci along with a slight change of enterococci in comparison with the use of 0.5 ml/kg dose. The information about the benefits of certain dose use and their maximum values of microbial agents, as V.M. Bondarenko and V.G. Petrovskaya emphasize [30] point to the need of microecological adequacy rule observance. It is obvious that microecological adequacy is the optimal ratio of the microbial intestine community [31].

CONCLUSION

The study of the toxic properties, teratogenic, embryotoxic, mutagenic, allergenic and carcinogenic activity of probiotic based on Lactobacillusplantarum strain VKPM B-2347 and Propionibacteriumfreudenreichii strain VKPM B-6561 in the nutrient substrate showed that the drug has the following properties:

- Does not cause the death of laboratory animals with its single and multiple injections into an animal body through the stomach and skin;
- Does not affect the animal reproductive function and fertility;
- Does not have teratogenic and embryotoxic activity;
- Does not have mutagenic properties concerning microorganisms and sex cell of animals;
- It has weak immune-stimulating properties;
- Does not have carcinogenic activity;

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- Has weak allergenic properties.

The obtained results of the drug oral administration to rabbits allow to characterize the suspension properties from L.plantarum and Pr.freudenreichii: makes an antagonistic effect and reduces the colonization potential of Staphylococcusssp., Salmonella, Shigella, Proteus, improves the growth of Lactobacillussp. and Bifidobacteriumssp. bacteria, as a result of the propionic acid bacteria symbiotic interaction.

SUMMARY

From the stated above info one may make the conclusion on the potential possibilities of Propionibacteriumfreudenreichii VKPM B-2347 and B-6561 Lactobacillusplantarum VKPM V-6561 strain use in rabbit breeding as a probiotic of curing and preventive action and the safety of their use.

Table 2. The sensitivity of rabbit gut microbiocenosis to Lactobacillus and Propionibacterium

Groups of microorganisms	Experiment start			10-th day of experiment			20-th day of experiment			30-th day of experiment		
	Contr.	1 gr.	2 gr.	Contr.	1 gr.	2 gr.	Contr.	1 gr.	2 gr.	Contr.	1 gr.	2 gr.
Bifidobacterium ssp., CFU/g	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^{10}*	10^{10}*
Lactobacterius ssp., mln. CFU/g	25,53 ± 4,04	27,57 ± 2,68	27,3 ± 4,51	80,25 ± 5,3	97,83 ± 29,3	93 ± 10,62	43,125 ± 5,46	75,15 ± 13,16**	75,1 ± 11,7**	45,075 ± 12,83	62,5 ± 12,63*	78,73 ± 10,41*
Enterococcus ssp., mln. CFU/g	26,285 ± 2,47	27,325 ± 3,82	24,6 ± 4,17	24,475 ± 1,75	31,075 ± 3,01*	24,25 ± 2,53	30,925 ± 5,06	26 ± 2,59*	26,25 ± 2,13*	30,8 ± 4,24	26,175 ± 5,03*	24,025 ± 2,52*
Enterobacteriaceaessp.												
Lactose negative, thous. KFU/g. (Salmonella, Shigella, Proteus)	229,75 ± 18,19	228,5 ± 30,51	259,75 ± 28,21	222,25 ± 21,792	190,75 ± 27,15*	195,75 ± 20,99	187,5 ± 11,82	168,25 ± 19,40*	223 ± 9,47*	206,25 ± 14,02	162,25 ± 19,37**	186,5 ± 17,95*
Lactose positive (Escherichia, Klebsiella, Citrobacter, Enterobacter), thous. KFU/g.	219,75 ± 21,05	235,25 ± 19,63	242 ± 34,1	246,75 ± 27,695	223,5 ± 10,15	220 ± 31,71	227 ± 22,04	209,5 ± 7,77	206,25 ± 12,33	210,75 ± 20,95	212,25 ± 4,89	214,75 ± 6,87
Staphylococcus ssp., $\times 10^4$ KFU/g.	226,6 ± 27,95	222,98 ± 23,24	209,025 ± 23,697	198,2 ± 9,08	191,7 ± 25,02	198,475 ± 22,54	144,1 ± 11,03	129,95 ± 5,45*	136,7 ± 7,71*	102,7 ± 5,46	94,75 ± 2,81*	98,4 ± 6,002*
E.coli haemolytic, thous. KFU/g.	54,75 ± 12,1	55,5 ± 18,42	62 ± 14,42	21,25 ± 6,32	15,75 ± 3,28*	16,75 ± 3,73*	2 ± 0,91	0***	0***	0	0	0
Candidassp., thous. KFU/g.	38,25 ± 9,59	30,25 ± 6,96	40,5 ± 13,53	5,75 ± 3,28	7,25 ± 4,75	5,5 ± 3,57	0	0	0	0	0	0

P ≥ 0,05; * P ≤ 0,05; ** P ≤ 0,01; *** P ≤0,001; KFU – colony forming unit

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Table 3. Changes of rabbit gut microbiocenosis after the exposure to Lactobacillus and Propionibacterium

Groups of microorganisms	60-th day of experiment			90-th day of experiment		
	Contr.	1 gr.	2 gr.	Contr.	1 gr.	2 gr.
Bifidobacteriumssp., KFU/g	10^9	10^{10}*	10^{10}*	10^{10}	10^{10}	10^{10}
Lactobacterius ssp., mln. KFU/g	42,65 ± 3,58	54,45 ± 5,17*	58,05 ± 6,17*	40 ± 5,71	44,225 ± 5,09*	44,475 ± 4,04*
Enterococcus ssp., mln. KFU/g	31,15 ± 3,48	28,725 ± 4,08	24,875 ± 3,1*	16,85 ± 2,04	15,2 ± 2,09	14,925 ± 2,39
Enterobacteriaceaessp.						
Lactose negative, thous. KFU/g (Salmonella, Shigella, Proteus)	184 ± 19,35	146 ± 20,03*	146,5 ± 18,53*	146 ± 18,899	143,25 ± 17,27	145,75 ± 10,87
Lactose positive (Escherichia, Klebsiella, Citrobacter, Enterobacter), thous. KFU/g	192 ± 6,18	198,5 ± 5,98	190,75 ± 4,59	210 ± 16,45	194,25 ± 5,62	191,75 ± 8,199
Staphylococcus ssp., × 10^4 KFU/g	97,575 ± 5,84	86,825 ± 4,03*	90,475 ± 3,63*	100,725 ± 6,59	83,4 ± 7,73*	83,475 ± 3,12*
E.coli haemolytic, thous. KFU/g	0	0	0	0	0	0
Candidassp, KFU/g	0	0	0	0	0	0

P ≥ 0,05; * P ≤ 0,05; ** P ≤ 0,01; *** P ≤0,001; KFU - colony forming unit.

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