БІОХІМІЯ, БІОТЕХНОЛОГІЯ, МОЛЕКУЛЯРНА ГЕНЕТИКА

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PROBIOTIC CORRECTION OF *DAPHNIA MAGNA* MICROBIAL PROFILE USING *LACTOBACILLUS CASEI* UCM 7280

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The anthibiotics use in aquaculture these days is severely restricted by European standarts of marketable fish products quality. According to this, one of the most efficient alternatives are probiotics. So the search of applicable probiotic microorganisms that can be applied in aquaculture is relevant. On the other side, probiotics introduction into the fish body is usually accomplished in composition with dry feed as a biofilm. But a lot of fish larvae are not capable of consuming dry artificial feed due to numerous reasons. For their transmission for endogenic nourishment from exogenic live feeds are used in aquaculture, which have a higher level of digestibility compared to granulated feed. One more advantage of live feed is that they can be used as vectors to provide the targeted delivery of probiotics into fish body. In this work Lactobacillus casei IMV 7280 was firsly tested for the needs of aquaculture. It is shown that the usage of this probiotic during the growing of carp whitebaits stimulates it growth and leads to unwanted microflora suppresion in water as well as in fish bodies. The optimal schedule of L. casei UCM 7280 bioencapsulation into live feed with Daphnia magna as an example is designed and suggested. It is shown that bioencapsulation procedure realization does not repress D. magna's normal mocrobiome, provides the increase of proteins and lipids level for 1,2 and 1,4 times accordingly and spends up the increasing of density level of fodder organisms.

Keywords: live feed, probiotics, bioencapsulation, Lactobacillus casei, Daphnia magna.

Introduction. Intensive technologies that are widely used in aquaculture these days together with increasing productivity of hidrobionts biomass production can be followed by the increasing danger of diseases. Using of antibiotics and different chemotherapeutic drugs is severely stricted these days by the European standarts of marketable fish products quality. According to this, one of the most efficient alternatives are probiotics (Dawood et al., 2019; Rodiles et al., 2018).

In gut microflora of the majority of freshwater fish with natural conditions of existence prevail aerobic gram negative bacteria *Pseudomonas, Enterobacter, Aeromonas, Acinetobacter* as well as gram positive *Bacillus*. Also anaerobic gram negative microorganisms *Vibrio* and gram positive *Clostridium* are available (Izvekova et al., 2007).

High antagonistic activity against a wide spectrum of pathogenic and conditionally pathogenic bacteria as well as high extacelullar enzymatic activity lead to the possibility of probiotic applying in aquaculture. As major factors of their anthagonism bacteriocynes, short-chained organic acids, hydrogen peroxide and anthibiotic substances should be highlighted. It is shown that in digestive system of healthy fish *Bifidobacterium longum, B. dentium, B. asteroids, Enterococcus faecium, E. hirae* and different species of *Lactobacillus* are present (Vlková et al., 2012; Araújo et al., 2015).

Probiotics introduction into the fish body is usually accomplished in composition with dry feed as a biofilm. But a lot of fish larvae on different development stages are not capable of consuming dry artificial feed due to numerous reasons. For their transmission from endogenic nourishment on exogenic live feeds are used in aquaculture that has a higher level of digestibility and assimilation compared to granulated fodder. Taking low level of fish larvae resistance on early ontogenesis stages into consideration, the question of their receiving of probiotics appears particularly acute. In this aspect we offered the procedure of *Lactobacillus casei* introduction in fish early larvae organisms through the live feed – zooplankton organism *Daphnia magna* that appears to be «live capsule» for probiotic introduction, while posessing high nutririon value (Bogut et al., 2010; Cheban et al., 2017). But before this technology can be introduced, the choose of optimal condition of bioencapsulation – effective dose, multiplicity of introduction, the term of saturation, control of mortality level and

nutrition value of folder organisms themselves should be selected.

Materials and methods. Study of probiotic correction of microbial profile of start live feeds was conducted using freeze-dried *Lactobacillus casei* UCM 7280, received from Danylo Zabolotny Institute of Microbiology and Virology of NAS of Ukraine.

Encapsulation of probiotic bacteria was conducted into the bodies of Cladocera organisms *Daphnia magna* Straus, 1820. Daphnia were cultivated on the standard medium ADaM (Artificial Daphnia Medium) in tanks with 0,5 *l* volume, with the beginning density 25 individuals per 0,5 liters. Zooplankton cultivation was conducted in the conditions of climate room with 16 hours photoperiod and temperature 22°C. As a control feed substrate for zooplankton water suspension of *Saccharomyces cerevisiae*, standardized to 24×10^6 CFU/l cells amount was used.

For the determination of the optimal bioencapsulation mode the next groups of experimental organisms were formed: control – daphnia that received yeast only as a main feed substrate; as well as experimental groups, that except from yeast received freeze-dried *Lactobacillus casei* UCM 7280 in amounts, that provided the forming of finish concentration $2,5\times10^5$ CFU/l (experimental group 1), 5×10^5 CFU/l (experimental group 2) and 10^6 CFU/l (experimental group 3). The first feeding and probiotic introduction was conducted right after forming the groups, the following ones were fed once per 48 hours. Daphnia cultivation was conducted during 7 days.

For the conduction of the microbiological research the homogenization of animal objects with the following sowing on the appropriate elective mediums was conducted. For the determination of general microbial landscape the homogenate was sowed on meat peptone agar (MPA), and on the MRS medium for the detection of *Lactobacillus* colonies. Besides the Gram stained fixed preparations were made. The amount of colony forming units were expressed per one *Daphnia magna* individual.

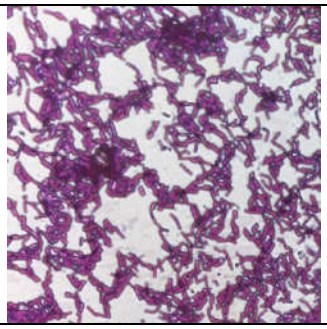
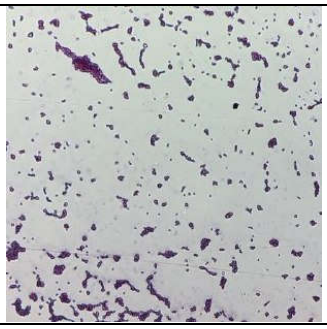
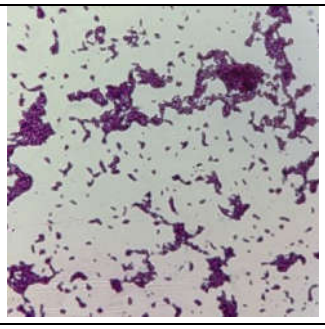
Results and discussion. It is obvious that bioencapsulation procedure has an influence on microbiome of organisms, used as live capsules while feeding larvae. During this autochthonous microflora of live feed will be replaced with a wated one with a different speed depending on the amount of applied probiotics, digestive system enzyme activity speciality, species composition of live feeds etc. But the integral criteria of successful procedure of bioencapsulation is the presence of needed microorganism in homogenates of fodder organisms sowing.

As known, filtrators' microbiome largely depends on environmental factors. Daphnias are not an exception. The basis of dygestive system microbiome of daphia are bacteria *Limnohabitans* sp. and other gram-negative rod-shaped bacteria. Normally lactic acid bacteria are not present in daphnia microbiome (Freese & Schink, 2011; Mushegian et al., 2019).

As the research showed, colonies of lactic acid bacteria were not present in control group samples. On the other hand, 3 types of colonies were noticed on the nutrient agar that is general purpose medium. They were all characterized with the superficial growth. As predicted, indigenous microflora of experimental culture of daphnia is presented with gram-negative (painted pink) rod-shaped bacteria (table 1). Futherly these cells were used as a reference compared to experimental groups´ microflora.

Table 1.

Morfological characteristics of Daphnia magna control group samples´ colonies that prevail on nutrient agar

Size	4 mm (large)	2 mm (average)	3,5 mm (average)
Edges	Wavy	Smooth	Irregular
Relief	Flat	Domed	Convexed
Structure	Homogenous	Homogenous	Homogenous
Colour	White	Yellow	White
View while microscopying			

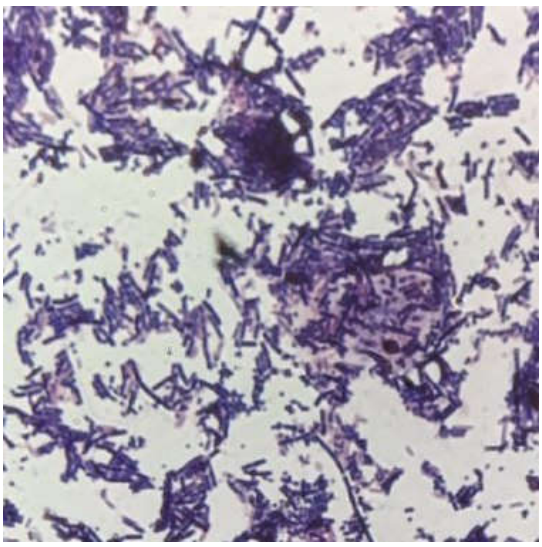


Fig. 1. Gram-positive rod-shaped Lactobacillus casei on MRS medium

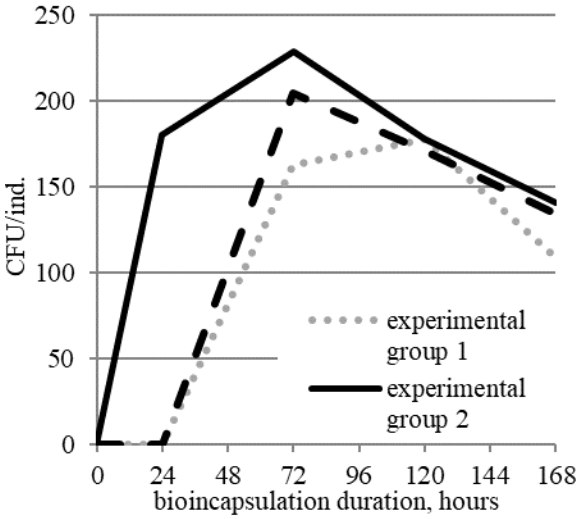


Fig. 2. The dynamics of D. magna colonisation with Lactobacillus casei depending on their concentration in the environment

While sowing control samples on MRS agar any *Lactobacillus*-like colonies were not present. Thus, after the introduction of experimental probiotics in the environment with daphnia, their microbiological landscape significantly differed from the one of control samples. It is known that on MRS agar *Lactobacillus casei* forms white round small- or average-sized colonies with smooth edges that grow inside the medium (Starovoitova et al., 2012).

Colonies like this were revealed in MRS-cultivated daphnias´ experimental samples homogenisates. They were averaged-sized colonies (D=1-3 mm) with the following characteristics: edge is a precise line, growth inside the agar, homogenous texture, white-coloured, with smooth outlines. The cells were rod-shaped, that is a characteristic of gram positive bacteria (fig. 1). All of the above let us to come to a conclusion anout lactic acid bacteria colonization of dysestive system of experimental fodder organisms.

The fastest saturation of daphnia with the experimental probiotics occurs while adding into the environment 5×10^5 CFU/l of lactic acid bacteria (experimental group 2). So, on the next day after this concentration of *L. casei* first introduction colonization features appeared. While applying other two studied concentrations of *L. casei* – $2{,}5\times10^5$ and 10^6 CFU/l (experimental groups 1 and 3 accordingly) the signs of colonization started to manifest just after the repeated introduction of *Lactobacillus* into the environment with *D. magna* (fig. 2). While applying all of the experimental concentrations, the largest amount of colony forming units of lactic acid bacteria accumulated in the bodies of daphnia after the twofold introduction of probiotics that is a third day of bioencapsulation.

Probiotic microflore is transitory and after the termination of its introfuction to the body usually starts to degrade slowly (Rodiles et al., 2018). Effect like this was noticed at fodder organisms after the discontinuation of bioencapsulation procedure – in 48 hours after the discontinuation of bioencapsulation procedure the amount of the *Lactobacillus* decreased significantly (fig. 3).

It is worth to be marked that the intensity of elimination of probiotic microorganisms depended on the initial level of their colonization of the hosts' body – the highest indicator of residual microflora was characteristic for daphnia of the 2-nd experimental group. It is obvious that the 5×10^5 CFU/l concentration of Lactobacillus provided the formation of the steadiest grouping in the zooplanktons digestive system.

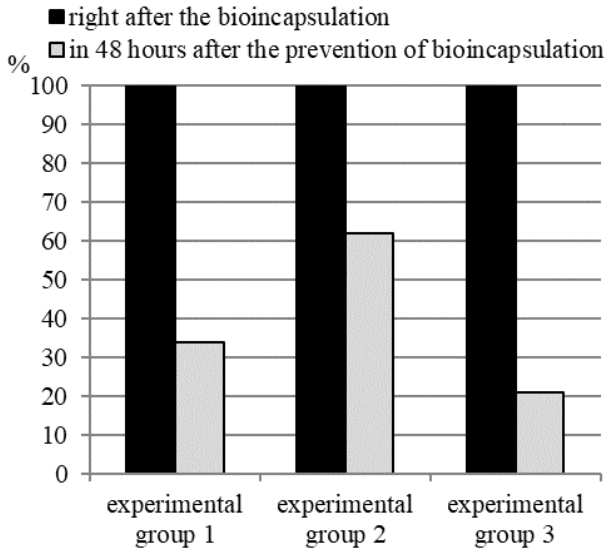


Fig. 3. Relative decreasing of amount of L. casei colonies during 48 hours after the termination of bioencapsulation procedure

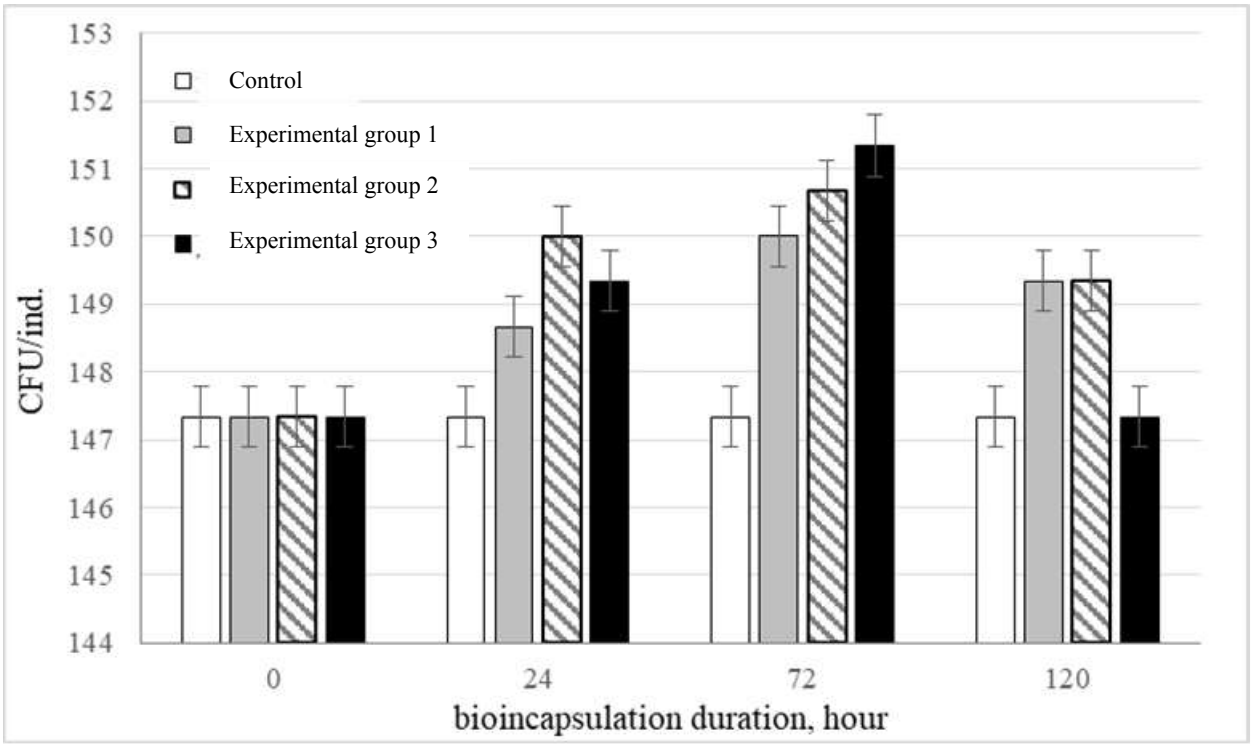


Fig. 4. The influence of L. casei bioencapsulation procedure on Daphnia indigenous microflora

As known, the positive influence of probiotics can appear not only as ponetially harmful microorganisms oppression, but also as indigenous microflora growth stimulation as the expense of growth-stimulating factors, vitamins synthesis, pH and redox potential normalisation etc (Thakur et al., 2016; Wang et al., 2017). For the estimation of the influence of procedure of bioencapsulation on daphnia microflora the counting of all experimental groups colonies grown on nutrient agar was conducted. The comparative analysis of the received results with control samples witnessed that saturation with probiotics does not repress the normal daphnia microflora (fig. 4).

Taking the received results into the account it can be summed up that for the conduction of the effective bioencapsulation of L. casei IMV 7280 into start live feeds 5×10^5 CFU/l of lactobacillus have to be added into the environment with them. The procedure of bioencapsulation itself is appropriate to be conducted for 3 days, with the double introduction of probiotic culture into the environment.

Conclusions. Therefore, the applying of *Lactobacillus casei* IMV 7280 while growing carp whitebaits promotes their growth acceleration for 13% as well as leads to repressing the unwanted microflore both in fish bodies and water they are nourished at. For the providing of targeted delievry of Lactobacillus into fish bodies on early tagged of their development it is appropriate to bioincapsulate them into live feeds. For effective *L. casei* IMV 7280 bioencapsulation conduction it is needed to provide the 5×10^5 concentraion of them. The procedure of bioencapsulation itself itself should be conducted for 3 days, with the double introduction of probiotic culture into the environment – at the beginning and repeatedly in 48 hours. The bioencapsulation conduction does not repress normal D. magna microflora, provides increasing of proteins and fats levels in 1,2 and 1,4 times accordingly and accelerates the growth of live feeds dencity.

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ПРОБІОТИЧНА КОРЕКЦІЯ МІКРОБНОГО ПРОФІЛЮ *DAPHNIA MAGNA* З ВИКОРИСТАННЯМ *LACTOBACILLUS CASEI* УКМ 7280

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Застосування в аквакультурі антибіотиків на сьогодні жорстко обмежено європейськими нормами щодо якості товарної рибної продукції. Відповідно, однією з найбільш дієвих альтернатив є застосування пробіотиків. Виходячи з цього актуальним є пошук дієвих пробіотичних мікроорганізмів, які б могли бути використані для потреб аквакультури. З іншого боку, введення пробіотика в організм риби переважно здійснюється безпосередньо у складі сухого корму у вигляді біоплівки. Проте личинки багатьох видів риб на ранніх етапах свого розвитку в силу цілого ряду причин не здатні споживати сухий штучний корм. Для переведення личинок з ендогенного живлення на екзогенне в аквакультурі використовуються живі корми, які мають набагато вищий рівень засвоюваності у порівнянні з гранульованими кормами. Ще однією перевагою використання живих кормів є те, що кормові організми можуть бути використані як вектори для забезпечення цільової доставки пробіотиків в організм личинок риб. У роботі вперше апробовано застосування Lactobacillus casei УКМ 7280 для потреб аквакультури. Показано, що застосування даного пробіотику при вирощуванні мальків коропа сприяє прискоренню їх росту, а також призводить до пригнічення небажаної мікрофлори як в організмі риб, так і у воді. Розроблено та запропоновано оптимальний режим біоінкапсуляції L. casei УКМ 7280 в живі корми на прикладі монокультури Daphnia magna. Показано, що проведення процедури біоінкапсуляції не викликає пригнічення нормальної мікрофлори у D. magna, забезпечує підвищення рівня накопичення живими кормами білків та ліпідів в 1,2 та 1,4 рази відповідно, стимулює пришвидшує темпи наростання щільності культури кормових організмів.

Ключові слова: живий корм, пробіотики, біоінкапсуляція, Lactobacillus casei, Daphnia magna.

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