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JOINT CULTIVATION OF REPRESENTATIVES OF THE GENERA *PROPIONIBACTERIUM, LACTOBACILLUS, BIFIDOBACTERIUM* ON UNIFIED MEDIA

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Probiotic microorganisms are living non-pathogenic, non-toxigenic microorganisms that enter the intestine with food and have a positive effect on the body, normalising the composition and biological activity of the microflora of the digestive tract. Lactic acid bacteria of the genera Lactobacillus and Bifidobacterium occupy a prominent place among the microorganisms that make up probiotics. The presence of these bacteria in the gastrointestinal tract provides an antagonistic environment that is directed against pathogenic microorganisms and creates conditions for the growth and development of beneficial bacteria. Bifidobacterium and Lactobacillus can enhance the absorption capacity of food components, lactose tolerance, stabilise liver function by normalising bowel function and reducing blood ammonia levels.

Recently, the spectrum of microorganisms that exhibit antagonistic properties and have a favourable effect on the vital activity of the organism has been expanding. Much attention is paid to propionic acid bacteria, which are part of the microflora of various biotopes. Propionic acid bacteria synthesise vitamin B12, bifidogenic growth factors and bacteriocins, have high adhesive and immunomodulatory properties, and their metabolites have antimutagenic effects. In this study, we analysed the possibility of individual and joint cultivation of representatives of the genera Propionibacterium, Lactobacillus and Bifidobacterium on unified media containing corn and yeast extracts. It was found that these nutrient media are effective for monocultivation of propionic acid bacteria and lactobacilli. At the same time, not only an increase in the optical density of propionic acid bacteria (3-fold), but also an increase in acid formation (2-fold on average) was observed. The possibility of joint cultivation of lactic acid and propionic acid bacteria by creating associations of two types was proved. The number of colonies of the studied microorganisms on selective nutrient media after cocultivation in unified media of both types was determined, the highest survival rate was observed for Propionibacterium. The antagonistic effect of both individual cultures and established associations against certain gram-positive bacteria and microscopic fungi was recorded.

Keywords: Propionibacterium, Lactobacillus, Bifidobacterium, associations, cocultivation, unified nutrient medium.

Introduction. A probiotic is a functional food ingredient in the form of microorganisms useful for humans or animals (most often representatives of the genera *Bifidobacterium, Lactobacillus* and associated with them), which, when systematically consumed, provides a beneficial effect on the body due to the normalisation of the composition and/or increase in the biological activity of the normal intestinal microflora (Feng P. et.al., 2018; Amara A. A., Shibl A. M., 2015). The relevance of the task of using probiotics is confirmed by the high frequency of dysbiotic disorders of the intestinal microbiocenosis, which are one of the most common causes of maladaptation.

Probiotics act at three levels: in the intestinal cavity (compete with pathogenic and opportunistic microbiota); only at the level of the intestinal epithelium (increase the effects of the protective intestinal barrier); at the level of intestinal immunity (have an immunomodulatory effect). The following effects of probiotics are observed: participation in the biochemical mechanisms of digestion and competition for food components with pathogens; changes in local pH and other metabolic characteristics of the internal environment; production of bacteriocins; neutralisation of superoxide radicals; stimulation of epithelial mucin production; enhancement of intestinal barrier function; competition with pathogens for adhesion; modification of pathogenic toxins; stabilisation of the microbial community (de Vos W. M. et.al, 2022; Fukuda S. et.al., 2011).

At the same time, there is growing evidence that the impact of symbiotic bacteria is not limited to the gut, but can extend to other organs (Wieërs G. et.al., 2020). To date, the exact mechanism of action of probiotics remains undisclosed. It is likely that the positive effect of probiotics is manifested through several mechanisms.

The use of probiotics in animal husbandry, in particular in aquaculture, is of great importance (Hasan K.N., Banerjee G., 2020). The industrial artificial cultivation of aquatic organisms in closed water supply systems with high planting density often provokes an increase in the level of organic water pollution, as well as instability of dissolved oxygen concentration and an increase in the number of opportunistic bacteria in the entire hydroecosystem. These factors lead to the development of stress in animals with a subsequent decrease in their immunity and loss of resistance to pathogens of various diseases (Hoseinifar S. H. et.al., 2018). The use of antimicrobials for prevention and treatment is not always effective. In addition, antibiotics often result in a deficit of beneficial microflora, the gradual development of pathogen resistance to the drug, and a decrease in immune response. The negative impact of these factors on the productivity of fish farms is obvious (Wuertz S. et.al., 2021), so the use of probiotics is a significant modern alternative (Luna G.M. et.al., 2022; Talwar C. et.al., 2018).

The range of microorganisms that can act as probiotics is constantly expanding. In recent years, representatives of the genus *Propionibacterium* have attracted considerable attention. Classical ("lactic") propionic acid bacteria (PAB) have unique immunomodulatory and antimutagenic properties, they take root in the intestine and are able to reduce the genotoxic effects of a number of chemical compounds and UV rays (Deutsch S-M. et.al., 2017). It is known that the positive role of propionic acid bacteria is due to the production of propionic acid, minor organic acids, bacteriocins and enzymes, as well as the synthesis of large amounts of vitamin B12 (Piwowarek K. et.al., 2018). It is also worth noting the ability of live *Propionibacterium*, as well as *Bifidobacterium*, to biosorb heavy metals, which effectively bind cadmium and lead in the presence of food (Massoud R., Zoghi A., 2022; Halttunen T. et al., 2008). The same applies to the decontamination of cyanotoxins and mycotoxins, as well as the ability of propionic acid bacteria to bind plant lectins that cause harmful local and systemic reactions, including inducing the proliferation of colon adenocarcinoma cells (Zarate G., 2017). An important feature of radiopharmaceuticals is their ability to form bacteriocins, most of which are low molecular weight proteins with a molecular weight of less than 10,000 Da (Faye T. et.al., 2011).

Representatives of various species of classical propionic acid bacteria release proteins into the environment, including reactivating factors that can enhance or restore the viability of cells exposed to stressors (ultraviolet light, ethanol, heavy metal ions, heat). It is assumed that the mechanism of cell reactivation is associated with the induction of the synthesis of anti-stress proteins, primarily chaperones. The bacterial adaptation is also facilitated by the production of glycogen and trehalose (R. Ruhal et.al., 2013).

Propionic acid bacteria demonstrate in vitro tolerance to gastrointestinal inhibitory factors. When exposed to acids and bile salts, *P. freudenreichii* expresses general stress proteins involved in the cellular response to oxidative stress and DNA damage. These results have been confirmed by *in vivo* studies. *P. freudenreichii* targets its genome expression to use substrates available through the gut, such as propanediol, gluconate and lactate, to maintain its metabolism, which avoids starvation during transit (Frohnmeyer E. et.al., 2018). In addition to the ability to withstand lytic stresses, probiotic microorganisms must be preserved in the digestive tract to interact with host cells and produce the expected positive effects. The lifespan of probiotics in the digestive tract depends on the adhesion ability of the intestinal mucosa (in radioactive waste, provided by S-layer proteins and surface adhesins) and their growth rate.

All of the above indicates that propionic acid bacteria and their metabolites are an important factor in maintaining the balance of the microbial ecosystem of a macroorganism. The unique beneficial properties of PABs and their complete absence of toxicity argue for their inclusion in therapeutic and prophylactic agents for the protection and restoration of microflora.

Therefore, the aim of the study was to investigate the possibility of cocultivation of propionic acid and lactic acid bacteria by creating associations on unified complex media with corn and yeast extracts.

Materials and methods. Pure cultures of microorganisms (kindly provided to us by employees of the Institute of Microbiology and Virology named after D.K. Zabolotny National Academy of Sciences of Ukraine) and *Propionibacterium*, a culture isolated by us, were used for research.

Bacteria were monocultivated: *Lactobacillus* in MRS medium, *Bifidobacterium* in Blaurock medium, and *Propionibacterium* in the medium proposed by us (based on a thorough analysis of the component composition of the medium used for *Propionibacterium* cultivation), which contained magnesium chloride, sodium citrate, potassium phosphate, ascorbic acid, cobalt chloride, glucose, and peptone.

Also, for individual and cocultivation, modified unified media were used, which contained all the necessary components to ensure the normal growth and development of the studied microorganisms, namely peptone - 10 g/l, sodium acetate - 15 g/l, magnesium sulfate - 0.56 g/l, manganese sulfate - 0.12 g/l, potassium phosphate - 2 g/l, glucose - 15 g/l, cysteine - 0.5 g/l, ascorbic acid - 0.2 g/l. In addition, corn extract was added to one sample of the nutrient medium, and yeast extract to the other. Their content in both cases was the same.

Cultivation was carried out at 37 °C in 250 ml Erlenmeyer flasks, with a total working volume of 50 ml and an inoculum content of 10%.

The optical density of the cultures was assessed using a photocolourimeter at 540 nm. The control was the appropriate sterile culture medium.

The content of organic acids was determined by the titrimetric method, taking into account that 1 ml of 0.1 n NaOH corresponds to 0.009 g of lactic acid, and 1 ml of 1 n NaOH corresponds to 0.06 g of acetate.

To create an association of the first type, we used inoculum of microorganisms grown on a unified complex medium. The main fermentation process lasted 24 hours at 37 °C. Unified nutrient media and 5% inocula of *Propionibacterium*, *Bifidobacterium* and *Lactobacillus* cultures were added to 250 ml Erlenmeyer flasks.

To create associations of the second type, each individual culture was first grown on the appropriate medium: *Lactobacillus* in MRS liquid nutrient medium, *Bifidobacterium* in Blaurock liquid medium, and *Propionibacterium* in modified medium. Subsequently, the daily cultures were used to create associations and co-culture in standardised nutrient media. The cultivation conditions are identical to those described above.

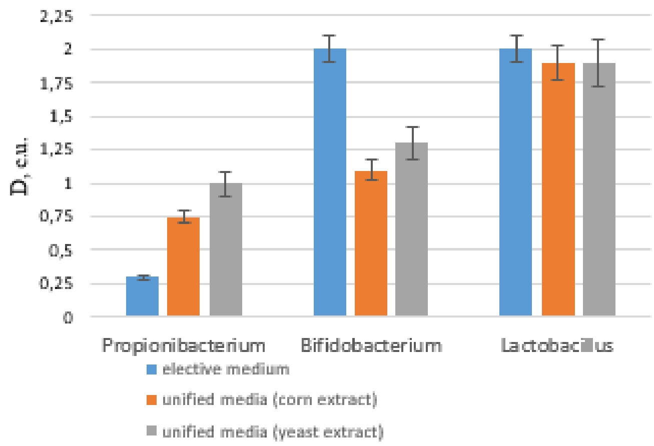
To test the viability of the studied microorganisms, they were sown on selective agarified nutrient media for each culture. Incubation was performed overnight at 37 °C and the number of colonies formed was counted.

The method of delayed antagonism was used to assess the antagonistic properties of the studied monocultures and associations. The culture medium was MPA. The test cultures were: gram-positive *Bacillus subtilis*, *Corynebacterium glutamicum*, *Enterococcus faecalis*, *Micrococcus luteus*, gram-negative bacteria *Pseudomonas syringae* and microscopic fungi – *Rhodotorula glutinis*, *Rhodotorula minuta*, *Rhodotorula rubra*, *Saccharomyces cerevisiae*.

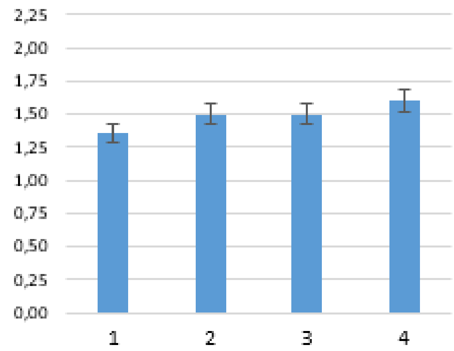
The results of the experimental data were processed statistically using Microsoft Excel software. The results were considered reliable at the level of confidence $p \leq 0.05$ according to the Student's criterion.

Results and discussion. At the first stage of experimental studies, the development of probiotic cultures was evaluated under conditions of monocultivation in selective and unified nutrient media. The experimental data showed the following (Fig. 1): density indicators largely depend on both the component composition of the nutrient medium and the culture being grown; when growing *Lactobacillus*, active development was recorded on the standard medium and no significant difference in indirect indicators of lactobacillus biomass accumulation was recorded on both complex unified media; representatives of the genus *Bifidobacterium* demonstrated active growth on Blaurock medium, while the amount of biomass formed on complex media was significantly lower; the total biomass of propionic acid bacteria in the selective medium was insignificant, while in the complex unified media it increased.

Density indices were also evaluated for associations. We did not find any significant differences in all 4 cases studied.



A



B

Fig. 1. Indicators of optical density under the conditions of mono- (A) and cocultivation (B) of Lactobacillus, Bifidobacterium and Propionibacterium

Note: 1 – association in medium with corn extract (inoculum – uniform medium), 2 – association in medium with corn extract (inoculum – elective medium), 3 – association in medium with yeast extract (inoculum – uniform medium), 4 – association in a medium with yeast extract (inoculum – elective medium)

The results of the experimental studies showed ambiguous trends in the formation of short-chain fatty acids when bacteria were cultivated in different media (Fig. 2). PAB and *Lactobacillus* converted the substrate into organic acids more actively when grown in unified media. The opposite patterns were recorded for *Bifidobacterium*. The amount of lactate produced by *Bifidobacterium* on unified media was 2.5-2.7 times lower on average compared to Blaurock medium.

The experimental data indicate that *Propionibacterium* produced significantly more acetate in complex media with corn and yeast extracts, respectively, compared to the selective medium. The amount of acetic acid produced by bifidobacteria in the unified media was lower than in the selective media. Representatives of the genus *Lactobacillus* produced more acetate on complex media compared to the selective medium (Fig. 2). It is known that propionic acid bacteria fermentation produces mainly propionic and acetic acids, to a lesser extent lactic and succinic acids, acetoin, diacetyl, and other volatile aromatic compounds such as dimethyl sulfide, acetaldehyde, propionic aldehyde, ethanol, and propanol. The organic acids synthesised by probiotic cultures – lactate, acetate and, obviously, propionate – have a pronounced antimicrobial effect against pathogenic microorganisms and form a favourable pH environment for autochthonous microorganisms. During the development of *Propionibacterium*, the ratio of propionic acid to acetic acid can vary widely, usually calculated as 2:1.

The study of lactic acid content in the cultivation of associations of two types showed no significant differences. The unified medium with corn extract was more efficient for the production of acetate by microorganisms; it accumulated 1.25-2 times more acetate than the medium containing yeast extract (Fig. 2).

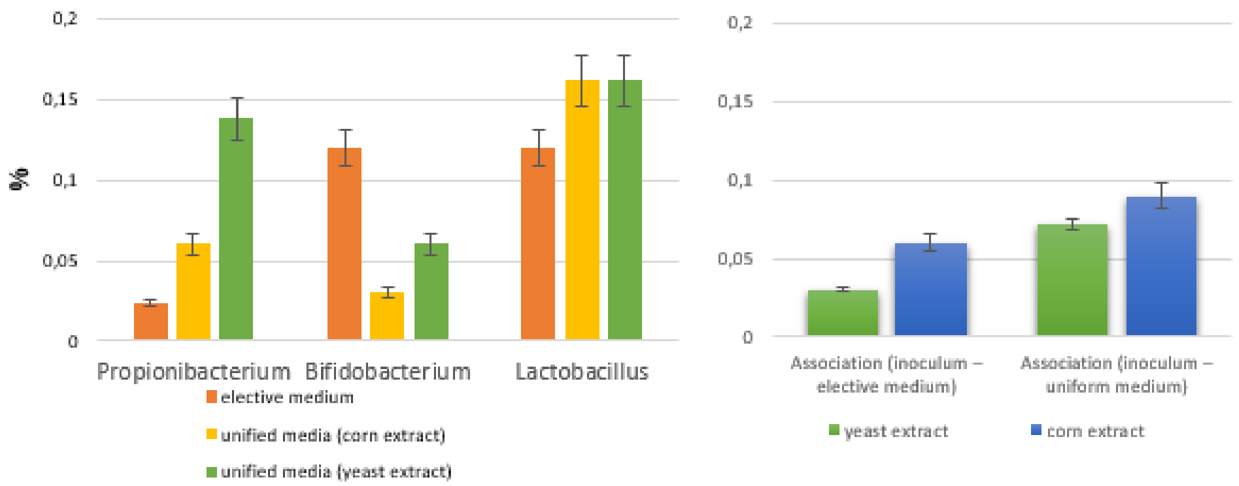


Fig. 2. Acetate content in the culture medium of the studied microorganisms

The assessment of the viability of microorganisms showed that the survival rate of bifidobacteria in unified media was reduced compared to monocultures. On the contrary, the number of live cells of propionic acid bacteria and lactobacilli in complex dense media was higher.

There are literature data on the cocultivation of *P. freudenreichii* and *Bifidobacterium longum*, which is manifested in a sharp increase in the antagonistic effect against such test microorganisms as *Micrococcus luteus* and *Staphylococcus aureus* due to the increased accumulation of organic acids in the nutrient medium (Taniguchi M. et.al., 1998). According to our experimental studies, in general, associations of probiotic bacteria have a significant inhibitory effect on the development of test cultures compared to monocultures (Fig. 3). Among the individual cultures, lactic acid bacteria were the most active antagonists. *Lactobacillus* and *Bifidobacterium* inhibited the growth of *R. rubra, C. glutamicum, M. luteus*, while *Propionibacterium* caused the appearance of significant lysis zones in relation to *M. luteus*. The associations of both types showed antagonism against 6 test cultures: *B. subtillis, R. glutinis, R. minuta, R. rubra, C. glutamicum, M. luteus*. Their effect on *Saccharomyces cerevisiae, Enterococcus faecalis, Pseudomonas syringae* was indifferent. The development of *R. glutinis* was more effectively inhibited by associations of the second type of bacteria grown in the medium with yeast extract.

A *B* *C*

Fig. 3. Antagonistic effect of Bifidobacterium (A) and associations of probiotic bacteria (B – association in medium with corn extract (inoculate – uniform medium; C – association in medium with yeast extract (inoculum – uniform medium)) on the development of test cultures

Note: 1 – Micrococcus luteus, 2 – Enterococcus faecalis, 3 – Saccharomyces cerevisiae, 4 – Pseudomonas syringae, 5 – Bacillus subtilis, 6 – Rhodotorula glutinis, 7 – Rhodotorula minuta, 8 – Rhodotorula rubra, 9 – Corynebacterium glutamicum

Thus, the unified nutrient media proved to be effective for monocultures of propionic acid bacteria and lactobacilli, as well as for associations created on their basis. The associations of bacteria of the two types grown on these nutrient media showed an active antagonistic effect on a number of test cultures. The creation of unified nutrient media is an alternative to ready-made industrial media, which may not always be available.

Conclusions. The use of modified unified nutrient media containing corn and yeast extracts proved to be suitable for the co-cultivation of the studied probiotic microorganisms, as well as effective for the growth of propionic acid bacteria and lactobacilli. An increase in the content of organic acids (lactate and acetate) was recorded in the case of monoculturing *Propionibacterium* and *Lactobacillus* when using unified complex nutrient media. Individual cultures of *Lactobacillus*, *Bifidobacterium* and *Propionibacterium* were more active antagonists against *R. rubra, C. glutamicum, M. Luteus, B. subtillis*. The associations of both types showed a high inhibitory effect against 6 test cultures studied: *B. subtillis, R. glutinis, R. minuta, R. rubra, C. glutamicum, M. luteus.*

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СУМІСНЕ КУЛЬТИВУВАННЯ ПРЕДСТАВНИКІВ РОДІВ *PROPIONIBACTERIUM, LACTOBACILLUS, BIFIDOBACTERIUM* НА УНІФІКОВАНИХ СЕРЕДОВИЩАХ

Л. М. Васіна, Н. А. Штогрин

Пробіотичні мікроорганізми – це живі непатогенні, нетоксиногенні мікроорганізми, що надходять в кишечник з їжею та позитивно впливають на організм, нормалізуючи склад і біологічну активність мікрофлори травного тракту. Молочнокислі бактерії родів Lactobacillus та Bifidobacterium посідають чільне місце серед мікроорганізмів, які входять до складу пробіотиків. Присутність даних бактерій у шлунково-кишковому тракті забезпечує антагоністичне середовище, яке направлене проти патогенних мікроорганізмів, створює умови для росту і розвитку корисних бактерій. Bifidobacterium, Lactobacillus здатні посилювати абсорбційні можливості харчових компонентів, переносимість лактози, стабілізувати функцію печінки за рахунок нормалізації роботи кишечника та зниження рівня аміаку в крові.

Останнім часом розширюється спектр мікроорганізмів, що виявляють антагоністичні властивості та чинять сприятливий вплив на життєдіяльність організму. Значну увагу приділяють пропіоновокислим бактеріям, які входять до складу мікрофлори різних біотопів організмів. Пропіоновокислі бактерії синтезують вітамін B12, біфідогенні фактори росту та бактеріоцини, володіють високими адгезивними й імуномодулюючими властивостями, їх метаболіти виявляють антимутагенну дію. У роботі проаналізували можливість індивідуального та сумісного культивування представників родів Propionibacterium, Lactobacillus та Bifidobacterium на уніфікованих середовищах, що містили кукурудзяний та дріжджовий екстракти. Встановлено, що дані поживні середовища є ефективними для монокультивування пропіоновокислих бактерій та лактобацил. При цьому відзначали не лише збільшення показників оптичної густини пропіоновокислих бактерій (у 3 рази), а й посилення кислотоутворення (в середньому у 2 рази). Доведено можливість сумісного культивування молочнокислих та пропіоновокислих бактерій шляхом створення асоціацій двох типів. Визначено кількість колоній досліджуваних мікроорганізмів на елективних поживних середовищах після кокультивування в уніфікованих середовищах обох типів, найвища виживаність відмічена для Propionibacterium. Зафіксовано антагоністичний вплив як індивідульних культур, так і створених асоціацій щодо окремих грампозитивних бактерій, мікроскопічних грибів.

Ключові слова: Propionibacterium, Lactobacillus, Bifidobacterium, *асоціації, кокультивування, уніфіковані поживні середовища.*

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