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THE INFLUENCE OF THE FOOD RATION ON THE PROCESS OF MULTIPLICATION AND DEVELOPMENT OF THE INTESTINAL *ENTEROCOCCUS* COMPONENT

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Abstract

The action of food rations on the process of multiplication and development of intestinal enterococci was tested in order to highlight their influence on the health of the intestinal tract. In the study were used four food rations with various caloric structure tested on laboratory animals - white rats, Wistar line. It was established that all the investigated rations differentially influenced the multiplication and development of enterococci. Based on the obtained results, it can be stated that the quantitative indices of enterococci, to a large extent, depend on the composition of food rations. Therefore, we consider that their numerical value can be regulated and maintained not only by microbial preparations with probiotic action but also by the use of food rations, which reflect the prebiotic influence of intestinal enterococci.

Key words*: genus Enterococcus, food rations, multiplication, streptococci.*

INTRODUCTION

Food and its components largely determine the health status of the human organism, in particular through the action on the intestinal microflora.

The intestinal microflora or microbiota is an indispensable component of the digestive tract, being represented by all microorganisms (bacteria, archaea, unicellular eukaryotes like fungi and protozoa, etc.), which through its activity produce various substances / molecules necessary for the normal activity of the human and animal organism (Rowan-Nash et al., 2019).

In general, the intestinal microflora comprises about 50 genera and several hundred species (from 300 to 1000) (Eckburg et al., 2005; Frank et al., 2007). Of these species, only 30-40 constitute the majority (99%) of intestinal bacteria (Sears, 2005). Over 99% of intestinal bacteria are anaerobic, but in cecum, aerobic bacteria reach high densities (Sherwood et al., 2013).

The bacteria of the intestinal microflora are divided into four phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, most of which are attributed to the genera: *Bacteroides*, *Clostridium*, *Faecalibacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus* and *Bifidobacterium*, and a little part to *Escherichia* and *Lactobacillus*. Numerically dominant bacterial genera are considered to be of major importance in the functioning of the host organism (Khanna et al., 2014).

The concentration and composition of the microbiota varies along the gastrointestinal tract (gut). Only a few species of bacteria are reported in the stomach and small intestine. The large intestine and colon are the most densely populated "habitat" of microorganisms, which can compete with any ecosystem on earth: about 10^{12} microorganisms per gram of intestinal contents (Guarner & Malagelada, 2003; O'Hara & Shanahan, 2006).

The functions of the microbiota are multiple, and its significance is proven by new studies, which demonstrate the close connection between intestinal bacteria and many diseases or conditions we face. The role of the intestinal microbiota in regulating homeostasis, in metabolism, absorption of vital nutrients and synthesis of vitamins, in the formation of immunity, and of the mechanical barrier, which protects the body from harmful agents, has been proven. In general, the intestinal

microflora has multiple physiological roles with resonance on the whole host organism (Hord, 2008).

Observational results over the past two decades suggest that the intestinal microbiota may contribute to the metabolic health of the human host and, and when it is aberrant - to the pathogenesis of various common metabolic disorders, including obesity, type 2 diabetes, non-alcoholic liver disease, cardio and metabolic diseases and malnutrition (Fan & Pedersen, 2021).

The role of the microbiota in the maturation of the immune system was also elucidated, being described the mechanisms of induction and ensuring the functioning of the immune system of the host organism by the intestinal microorganisms (Belkaid & Hand, 2014; Cianci, 2018).

The intestinal microbiota has an essential role in the solubilization of undigested and unabsorbed food residues and their elimination from the body, as well as in the protection against various pathogens by neutralizing toxic compounds (Sekirov et al., 2010; Griffiths, 2015). Intestinal bacteria are a crucial component of the enterohepatic circulation which in turn can influence the metabolization of many drugs, including antibiotics (Gorbach, 1996).

It has recently been shown that by regulating the level and composition of autoantibodies related to appetite-regulating hormones, the microbiota controls aspects of appetite-related behavior and the pathophysiology of eating disorders (Lam et al., 2017).

Consequently, dysbiosis, i.e., qualitative and functional impairment of the intestinal flora, is a serious avenue for understanding the cause of certain disorders, particularly those with underlying autoimmune or inflammatory mechanisms. This has become a central theme in biological and medical research.

On the other hand, the significance of intestinal bacteriocenosis consists in the production of substances (compounds) which in turn have a positive or negative effect not only on the digestive system but also on the whole organism (Gibson and Roberfroid, 1994).

The contribution of the microbiota in the production of various substances, essential amino acids, vitamins, especially B-complex vitamins, necessary for the proper functioning of the host organism, is known. Its role in the production of substances such as dopamine, serotonin or other neurotransmitters, the intestinal microbiota, has also been elucidated, proving the possibility of the microflora to act at a distance (Mangiola, 2016; Swanson, 2015).

The role of protection or biological barrier has been established, by producing short-chain fatty acids (SCFA) and stimulating epithelial regeneration (Belkaid and Hand, 2014; Cianci, 2018). Extracellular metabolites of short-chain fatty acids (SCFA) excreted by the intestinal microbiota have been reported to play an important role in the regulation of intestinal homeostasis. In addition to providing energy, SCFA also causes immune stimulation in animal and human cells (Nakkarach et al., 2021).

There are data on the influence of microorganisms, especially intestinal microorganisms, on the metabolism of the host organism. The microbiota has enzymes that are not encoded in the human genome, but which are needed to perform physiological tasks or to supplement the action of digestive enzymes to break down substances such as polysaccharides, polyphenols. According to this, they can regulate the body's energy balance and cellular metabolism (Baghbani et al., 2020).

The primary role in the digestion of ingested nutrients belongs to the small intestine, as the first region in which ingested food components are subjected to the action of intestinal bacteria, and is the region that is predominantly involved in the digestion and absorption of primary nutrients (Booijink et al., 2007; Leser & Molbak, 2009).

Thus, the microbiota of the small intestine has a major importance for the host (Zoetendal et al., 2012) and an important influence on the physiology and health of the host organism (Cotter, 2011; Duerkop et al., 2009).

Streptococcus and *Veillonella* spp. are the predominant components among the bacterial populations of the small intestine (Bik et al., 2006). *Streptococcus* species are involved in the fermentation of sugars, producing lactic acid as the predominant final fermentation product. In turn, *Veillonella* are famous for

their ability to use lactic acid as a source of carbon and energy (Ng & Hamilton, 1971).

During the past century, the classification of the genus *Streptococcus* has been refined, with the most significant change occurring in 1984 when some species of bacteria of the genus *Streptococcus* were separated into two genera: *Enterococcus* and *Lactococcus* and most members of the Group D streptococci, including *Streptococcus faecalis* and *Streptococcus faecium*, were included in the new genus *Enterococcus* (Schleifer & Kipper, 1984).

The genus *Enterococcus* includes lactic acid bacteria - Gram-positive cocci with high potential for colonization of various habitats, including the digestive tract in animals and is characterized by increased resistance to extreme pH values, ionizing radiation, osmotic and oxidative stress, at high concentrations of heavy metals and antibiotics, as well as at temperatures up to 45°C (Vu & Carvallo, 2011). Only *E. faecalis*, *E. faecium*, *E. avium*, and *E. durans* can colonize the human intestine, but two most common species are *E. faecalis* (90-95%) and *E. faecium* (5-10%) (Ramsey M. et al., 2014).

Enterococci are a model for studying the influence of food rations on the intestinal microflora and how the food consumed can contribute to the health of the digestive tract and the host organism through their action on the microbiota (Tannock & Cook G., 2002). They are also used in studies on how the body copes to coexist with a variety of beneficial and harmful strains of the same species, probably managing to select the ones that are more advantageous. These bacteria, as highly evolved commensals, have been extensively used in the food industry and as probiotics to prevent or ameliorate disease (Ramsey et al., 2014; Sánchez et al., 2019). *Enterococcus* is therefore a good model of how certain diets can protect the host, promoting, or not, the growth of strains with different levels of safety once they have reached the intestine (Penders et al., 2006; Timoşco et al., 2015).

Based on the above mentioned, it is proposed that it would be rational to use intestinal enterococci in the development of new preparations for probiotic use.

Thus, the aim of the paper was to study the action of different food rations (developed for the first time in the Institute of Physiology and Sanocreatology, Republic of Moldova) on the process of multiplication and development of intestinal enterococci, which are of interest to the food and pharmaceutical industry.

MATERIALS AND METHODS

In order to reveal the impact of the new developed food rations on the multiplication and development of intestinal streptococci, two experiments were performed. In both experiments, the new food rations elaborated for first time at the Institute of Physiology and Sanocreatology were tested. The structure of the developed rations is reflected in Table 1.

Table 1. Caloric structure of newly developed rations, **%**

Basic indices	Variants of food rations					
	1	*2*	*3*	*4*	*5*	*6*
Proteins	8	9	10	11	12	14
Lipids	35	33	31	29	27	25
Carbohydrates	57	58	59	60	61	61

The first experiment was performed *in vitro* and aimed to highlight the rations that have a more pronounced effect on enterococci development and multiplication. This experiment included seven lots, being tested 6 new food rations developed, as follows: Lot I - control, in which the inoculation of enterococci was performed separately on nutrient medium Enterococco Agar (Aesculin Azide Agar Balls) (https: //assets.thermofisher.com/TFS-Assets/LSG/manuals/IFU1194.pdf).; lots II-VII - experimental lots, in which the inoculation of enterococci was performed together with the decoction of six food rations (Table 1).

The second experiment was performed *in vivo* using laboratory animals (white rats, Wistar line). In this experiment, the action of food rations (preventively selected in the *in vitro* experiment) on the process of multiplication and development of intestinal *Enterococcus* bacteria was tested. The structure of the experiment is as follows:

- Lot I - control (administration of food ration no. 1);
- Lots II-VII - experimental (administration of food rations no. 4, 5 and 6).

For this purpose, samples of intestinal (rectal) contents were collected from all animals in two stages: at the beginning and end of the experiments. The samples were subjected to

research using classical microbiological methods (Garmasheva & Kovalenko, 2010). Their inoculation was performed on agarized elective nutrient medium, recommended for enterococci (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/IFU1194.pdf).

Over 72 hours after incubation of the inoculated samples on Petri dishes at 37 ± 1ºC, quantitative indices of enterococci were calculated at 1 g of intestinal contents (by multiplying the number of colonies by diluting the sample). The final results are expressed in decimal logarithms (log) (GOST 30518-97, 2000).

RESULTS AND DISCUSSIONS

As above mentioned, food rations or diet have a direct action on the intestinal microflora. Modulation of nutrient concentrations in diets may have a differential influence on the intestinal microbiota, including *Enterococcus* species.

The results obtained in *in vitro* experiments (Table 2) reveal the differentiated action of the new food rations on the quantitative indices of enterococci.

Table 2. Numerical value of bacteria of the genus *Enterococcus* inoculated *in vitro* separately and in common with newly developed food rations

The lot	The way of inoculation *separately, **in common with 6 food rations	The amount of live microbial cells per 1 ml of suspension, logarithms decimal (log/ml)	The difference to control, %
I	*	8.69 ± 0.65	
II	**1	6.38 ± 0.39	-26.58
III	**2	6.63 ± 0.39	-23.70
IV	**3	6.17 ± 0.48	-28.99
V	**4	8.59 ± 0.63	- 1.15
VI	**5	8.77 ± 0.67	+ 0.92
VII	**6	8.50 ± 0.64	- 2.18

The obtained data demonstrate that the inoculation of enterococci separately on elective nutrient medium ensured their multiplication up to the quantitative level of 8.69 log/ml. The decoction of six variants of newly developed food rations contributed to obtaining of different results. Thus, in the lots, where were tested the rations with no. 1, 2, 3, which is characterized by a higher concentration of lipids, the number of microbial cells is lower compared to Lot I. Food ratios no. 4, 5 and 6 (with a higher concentration of proteins and carbohydrates) ensured a quantitative level of microbial cells identical to that of Lot I (control). It follows that the testes food rations acted on the process of multiplication of enterococci differently, ensuring various levels of development of these bacteria (from 6.17 to 8.77 log/ml) (Table 2).

It should be noted that in the performed experiments bacteria of the genus *Enterococcus* showed different sensitivity to the primary composition of food rations.

Thus, an inhibitory action on enterococci manifested food rations no. 1, 2 and 3. It was established that the ration no. 1 (containing 8% proteins, 35% lipids and 57%) and no. 3 (containing 10% proteins, 31% lipids and 59%) had the greatest effect of numerical inhibition of bacteria. The rations no. 4 and 6 also contributed, to a lesser extent, to the numerical reduction of these microorganisms. Food ration no. 5 (containing 12% proteins, 27% lipids and 61% carbohydrates) acted as a stimulant, contributing to the non-essential increase in the number of bacteria of this genus.

Therefore, based on the obtained results, it was found that the tested food rations had a different action on the process of multiplication and development of bacteria of the genus *Enterococcus*. The rations no. 1, 2 and 3 showed an inhibition action on microorganisms, and the ration no. 5 had a stimulating action on multiplication of bacteria. Thus, it can be stated that the quantitative indices of enterococci largely depend on the composition of food rations. Therefore, we consider that their numerical value can be regulated and maintained not only using the microbial preparations with probiotic action but also through the diet with different structure of components (nutrients), which reflect the prebiotic influence of intestinal enterococci.

In order to confirm the *in vitro* results, *in vivo* experiments were performed on white laboratory rats, Wistar line. For *in vivo* testing of the action of food rations on intestinal enterococci, rats were grouped into four experimental groups (lots). In the first lot, it was administered the food ration, containing 8% proteins, 35% lipids and 57% carbohydrates (ration 1); in lot II - the ration with the structure of 11% proteins, 29% lipids

and 60% carbohydrates (ration 4); in group III - the ration with the structure of 12% proteins, 27% lipids and 61% carbohydrates (ration 5) and group IV - the ration containing 14% proteins, 25% lipids and 61% carbohydrates (ration 6).

In general, the rations that showed action to stimulate the numerical growth of intestinal enterococci (in *in vitro* experiments) were selected.

The ration with the structure of 8% protein, 35% lipids and 57% carbohydrates served as a control (control lot).

The structure of the tested rations and the grouping of laboratory animals according to the experimental lots are indicated in Table 3.

Table 3. Quantitative characteristic of newly developed and *in vivo* tested food rations, %

Basic components	The quantity, %, according to the variants of the tested food rations/ number of experimental lots of animals			
	1/I	4/II	5/III	6/IV
Proteins	8	11	12	14
Lipids	35	29	27	25
Carbohydrates	57	60	61	61

The body mass of the experimental animals and the quantitative indices of *Enterococcus* were determined as a result of the tests.

The experimental data were noted at the beginning and end of the experiments (after 60 days of administration of food rations) and are reported in Tables 4 and 5.

Table 4. Body mass of rats used to experiment with various food rations

The lot	Weight of rats in g/1 animal, according to the time of determination		Weight gain g/1 animal	Difference to the beginning, %
	at the beginning	at the finally		
I	242.4 ± 17.96	325.2 ± 26.70	82.8	34.15
II	242.8 ± 13.49	356.2 ± 22.58	113.4	46.70
III	242.0 ± 15.36	368.8 ± 21.60	126.8	52.39
IV	242.8 ± 12.22	352.6 ± 29.64	109.8	45.22

The analysis of the data obtained on the body mass of the tested animals revealed the positive impact of the tested food rations, with varied nutritional value. The lowest increase in body weight (by 34.15%) during the administration of the tested rations was established in group I, which served as a control. The other variants of food rations tested (rations no. 4, 5 and 6) contributed to an increase in body weight, during their administration, respectively by 46.70%, 52.39% and 45.22%. Based on the data on body mass (weight gain) of laboratory animals, it was found that the most optimal tested ration proved to be food ration no. 5, which was administered to the animals in group III.

Next, the action of the tested rations on bacteria of the genus *Enterococcus* was determined, as a component part of the intestinal microbiota.

Based on obtained data (Table 5) it was established that the ration tested in group I (control) contributed to the increase by 69.27% of the final numerical indices of facultative microorganisms of the genus *Enterococcus*, which indicates the abundant development of these bacteria.

Table 5. The modification of the numerical indices of *Enterococcus* bacteria in the intestinal contents of rats, fed with different nutritional value food rations

The lot	The amount of microbial cells per 1 g of intestinal contents, decimal logarithms (log)		The difference, %	
	at the beginning	at the finally	Comparative to the beginning	Comparative to the lot I
I	5.11 ± 0.36	8.65 ± 0.42	+69.27	
II	5.50 ± 0.39	6.58 ± 0.48	+19.63	-23.93
III	5.67 ± 0.41	6.63 ± 0.39	+16.93	-23.35
IV	5.23 ± 0.22	6.17 ± 0.41	+17.97	-28.67

In the animals from experimental groups II, III and IV, during the administration of the tested rations, a non-essential increase of the number of microbial cells was observed. Thus, the numerical value of the researched bacteria increased respectively by 19.36%, 16.39% and 17.97%. However, compared to the animals from lot I, the numerical indices of enterococci decreased respectively with 23.93%, 23.35 % and 28.67%.

Consequently, the results obtained in *in vivo* conditions, when the food rations were testing on laboratory animals, do not confirm the data obtained in *in vitro* conditions, when food rations were testing on nutrient medium. The differences in the data obtained in *in vivo* and *in vitro* conditions, indicate that in the intestine of animals, bacteria of one or another kind of genera of obligative or facultative microorganisms do not act separately, but in

association. The antagonistic influence of the representatives of the intestinal microflora is most frequently manifested. In particular, enterococci are inhibited by *Lactobacillus* representatives. On the other hand, it is known that among intestinal enterococci, the species *E. faecalis* predominates quantitatively compared to *E. faecium,* and increasing of their number is not beneficial to the host organism.

The data regarding the action of diets on the gut microbiota are quite heterogeneous. This is largely determined by many factors such as the duration of diet administration, the structure of nutrients in food rations, the model organism studied and the types of analyzed microorganisms.

What is certain, is that the nutrient structure of the food rations has a direct action on the microbial composition (Li et al., 2009; Holscher et al., 2018; Johnson et al., 2019).

However, it is considered to have a positive impact those diets that contribute to maintaining the „ecological homeostasis" of intestinal microorganisms (Leeming et al., 2019).

Importance to maintain a constant level of enterococci derives from their property to produce a wide variety of bacteriocins often called enterocins. They are also active against Gram-positive foodborne pathogens, such as *L. monocytogenes* (Izquierdo et al., 2009). *E. faecium* and *E. faecalis* are the main producers of enterocins. Bacteriocin-producing probiotics could compete with intestinal pathogens for colonization or modulate the microbiota homeostasis (Salvucci et al., 2012; Cotter et al., 2013). Enterococci, due to the property of producing bacteriocins, can be used as a probiotic with beneficial effects on the health of the host organism.

Thus, the fact that the tested food rations, *in vivo* conditions, do not conduct to a large numerical increase of enterococci, indicates to their positive impact.

Therefore, we consider that their numerical value can be regulated and maintained not only through utilization of microbial preparations with probiotic action but also by using food rations, which reflect the prebiotic influence of intestinal enterococci (on the example of tested ration no. 5).

Thus, experimentally it was found that the dietary factor (tested food rations) during the entire investigation contributed to the optimization of the content of enterococci in the intestine of model animals.

CONCLUSIONS

It was found that the tested food rations show different action on the process of multiplication and development of intestinal enterococci.

The quantitative indices of enterococci depend to a large extent on the composition of food rations.

Among the tested rations, the ration with no. 5 had the best result, in terms of numerical modification of enterococci and based on data regarding the body mass of the tested animals.

Numerical value of enterococci can be regulated and maintained not only by microbial preparations with probiotic action but also by the use of food rations, which reflect the prebiotic influence of intestinal enterococci

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