Research Article

A Model for Synbiotic Activity Evaluation: Static and Continuous Co-Culture Fermentation of Bifidobacterium Adolescentis ATCC 15703 and Bacillus Cereus ATCC 9634

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Abstract. In this study, the ability of a probioticstrain (*Bifidobacteriumadolescentis*ATCC 15703) to inhibit the growth of the common food contaminant*Bacilluscereus*ATCC 9634was studied, both individually and as part of a synbiotic with FOS during batch or continuous fermentation (flow fermentation). The conditions of the flow fermentation corresponded to the parameters of the human large intestine: maintaining a pH of 6.8; anaerobiosis; and a medium flow rate of 0.04 h^{-1}. Bifidobacteria and bacilli were co-cultivated on a prebiotic carbohydrate substrate (10 g/L) and the prebiotic was replaced with glucose (10 g/L).The results of the batch and flow fermentation were compared.The synbiotic efficacy of the probiotic*Bif. adolescentis*and the prebiotic FOSagainst the common food contaminant*Bac. cereus*was shown for all conditions. Fermentation of a pure culture of bifidobacteria with varying prebiotic concentrations (2, 5, 10, 15 and 20 g/L) was carried out to study the state of dynamic balance. It was demonstrated that 48 hours is enough to achieve stable dynamic balance.Prebiotics were co-cultivated with varying carbohydrate concentrations of 5, 10, and 15 g/L.The results showed that increasing the prebiotic concentration increased the duration of the lag-phase and reduced the final number of bacilli.

Keywords: probiotics, prebiotics, synbiotics, gastrointestinal tract modeling, antagonism, co-culture fermentation

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1. Introduction

One of the main problems in developed countries is the failing of human health due to unfavorable environmental conditions, decreasing food quality, increasing stress levels, as well as lack of physical activity. It is necessary to pay special attention to daily human activity, namely nutrition to solve this problem. Based on research in the field of nutrition in the scientific community, as well as among the population, the concept of functional nutrition is gaining popularity, which includes natural products and their modifications to increase the nutritional value, positively affecting the prevention and treatment of diseases [1].

Particular interest has components of functional foods, such as probiotics, prebiotics, and synbiotics. Probiotics are viable microorganisms that provide benefits to the host when consumed in sufficient quantities [2]. Prebiotics are selectively fermentable ingredients that contribute to specific changes in the composition and activity of the gastrointestinal microflora [3]. Synbiotics include PRO - and prebiotics, while the prebiotic stimulates the probiotic. Based on this provide a synergistic effect. The complex effectiveness of synbiotic implies that the probiotic has a greater effect in the presence of a prebiotic than in its absence [4].

Probiotics act through a variety of mechanisms that affect the composition and function of the indigenous microbiota. The metabolic products of probiotics, namely, bacteriocins and short-chain fatty acids, exhibit antagonistic action against pathogenic and opportunistic microorganisms by interacting with their adhesion sites, suppress the synthesis of toxins [5]. Prebiotics stimulate the growth of indigenous bifidobacteria, in other words, they cause an improvement in the intestinal bifidoflora, reduce the amount of lipids in the blood and the time of passage through the gastrointestinal tract, reduce the risk of osteoporosis and diabetes mellitus [6]. Modern in vitro and in vivo studies have shown that the efficiency of synbiotic compositions in suppressing the growth of pathogens and food contaminants is higher than when using the components of functional nutrition separately [7-8]. The study of synbiotics in this direction is relevant and promising for the prevention and treatment of various diseases of the gastrointestinal tract, including diarrhea (especially diarrhea after antibiotic treatment), enterocolitis, rotavirus infection, pancreatitis, various intestinal inflammations and others [9].

To approximate the experimental conditions to real conditions, systems simulating the conditions of the gastrointestinal tract are successfully used. This is due to the fact that in vivo study is not always possible from an ethical and financial point of view. As an alternative to in vivo experiments, there are simple static models in vitro that simulate the gastrointestinal tract, but none of the static models reproduce the dynamic environment of the intestine. The preferred method of research is a dynamic model that allows scientists to regulate the pH, food flow, and injection of digestive enzymes in various parts of the gastrointestinal tract [10].To take into account every factor that affects the mathematical description of a dynamic model, it is necessary to use modern forecasting methods, such as neural networks. To create a neural network, you need to have a large amount of data to properly scale it [11]. Therefore, the final goal of the research is obtaining the results of probiotic and non-probiotic cultivation in vitro and evaluating the effect of prebiotic substances on the interaction of microorganisms in conditions close to the conditions of the gastrointestinal tract.

2. Materials and methods

2.1. Microorganisms, hemicalandreagents

The probiotic strain *Bifidobacteriumadolescentis*VKPM AC1662 (corresponds to ATCC 15703) and *Bacillus cereus* VKPM B8076 (corresponds to ATCC 9634, used in antibiotic activity tests) was purchased from Russian National Collection of Industrial Microorganisms (VKPM) have been used in this study. The strains were stored in freeze dried form at temperature not exceeding 4-8°C. Before usage, each sample was diluted and mixed with sterile 0.9% sodium chloride solution. The obtained suspension of each sample was transferred into 50 ml tubes, containing 25 ml of the cultural medium and incubated at 37 ± 1°C for 24-48 h. *Bac. cereus* was used as a test strain of a non-probiotic - an object of antagonistic activity, since it is a spore-forming soil bacterium and a common food contaminant [12].

FOS (Orafti ® P95, BENEO-ORAFTI, Belgium) was obtained from Bang & Bonsomer Russia was used as a prebiotic, glucose (Sigma) was used as a non-prebiotic substrate.

2.2. Fermentation

The fermentations were carried out under stationary conditions or in the flow cultivation mode.The medium with the following composition [13] was used for fermentations (g/L): casein triptone (Difco Laboratories) – 10; yeast extract (Springer) – 7,6; meat extract (Panreac) – 5; ascorbic acid (AppliChem) – 1; sodium acetate – 1; $(NH_4)_2SO_4$ – 5; urea – 2; $MgSO_4 \cdot 7H_2O$ – 0.2; $FeSO_4 \cdot 7H_2O$ – 0.01; $MnSO_4 \cdot 7H_2O$ – 0.007; NaCl – 0.01; Tween-80 – 1; cysteine – 0.5 (pH adjusted to 7.0 or 6,8 for flow fermentation).

The batch fermentation was carried out in the sealed vessel with two branches. Each of the branches was provided with the membrane autoclave vent filter (Midisart 2000 PTFE, 0.2 µm, Sartorius) and clamps. The medium was autoclaved for 30 min at 115 °C. FOS and glucose solutions were separately autoclaved and were added to the sterile medium to obtain a concentration of 10 g L^{-1}. The overnight cultures (16 h) of the bifidobacteria and bacillusstrains were used to inoculate the batch culture fermentations. The vessel was filled with N_2 (extra pure) through the inner branch immediately after inoculation. The fermentation was carried out at 37°C with shaking (180 rpm).

The flow fermentation was based on the “three-stage continuous” model, consisting of three fermentation vessels corresponding to certain parts of the gastrointestinal tract

[14]. Cultivation is carried out in one fermenter maintaining pH, temperature, atmosphere, flow rate and some other parameters characteristic of each department[15].

The study used a simplified model of the large intestine, consisting of a single bioreactorMinifors (Infors HT, Switzerland), as it describes the limiting stage of the process and facilitates research.The cultivation conditions corresponded to the large intestine: temperature 37°C, flow rate 0.04 h^{-1}, pH 6.8, anaerobiosis (argon bubbling to $pO^2 \leq 0.5$). For cultivation, an inoculate was used after two generations (24 hours for the first passage and 16-18 for the second).

2.3. SCFAs and lactic acid analysis

Acid concentration was measured using high performance liquid chromatography according to [16] and [17] with some modifications. HPLC was carried out to determine the content of organic acids such as lactic and acetic.HPLCwas performed on an Agilent 1220 Infinity LC liquid chromatograph with refractometric detection using a columnHi-Plex H (250×4,6 мм).

To construct a calibration graph, we used standard solutions of the determined organic acids (lactic, acetic), diluted with the mobile phase (the exact volume of sulfuric acid is 0.1 N and deionized water) in series.

2.4. Determination of bacterial cell growth

The bacterial counts were carried out in triplicate for each measure. *Bac. cereus*colonies were counted by plating on MRS medium [18] at 37 °C in air. Bifidobacteria colonies were counted on BFM medium [19] composed of (g L^{-1}): peptone 10, sodium chloride 5.0, lactulose 5.0, cysteine hydrochloride 0.5, riboflavin 0.01, yeast extract 7, meat extract 5, starch 2, thiamine chloride 0.01, lithium citrate 3.3. The pH was adjusted to 5.5 by adding of propionic acid (5 mL L^{-1}). The Petri dishes were incubated under anaerobic conditions provided by BD GasPak^{TM} anaerobic container system at 37 °C. The specific growth rate was calculated as the slope of log10 of the bacterial count in the exponential phase to time.

3. Results and discussion

As a result of batch fermentation of pure and mixed culture of probiotic and non-probiotic strains, growth curves of bifidobacteria (data not shown) and bacilli (Figure

Table 1: Parameters of pure and mixed *Bac. cereus* ATCC 9634 and *Bif. adolescentis* ATCC 15703 cultures growth.

Condition	Carbohydrate	Maximum specific growth rate, h^{-1}		Yield of probiotic, ng CFU^{-1}	
		Probiotic	Non-probiotic	Lactic acid	Acetic acid
In mixed culture	FOS	0,45	1,08	$2,09 \times 10^{-3}$	$3,09 \times 10^{-3}$
	Glucose	0,54	0,89	$7,66 \times 10^{-4}$	$2,44 \times 10^{-3}$
In pure culture	FOS	0,27	0,84	$1,32 \times 10^{-2}$	$1,39 \times 10^{-2}$
	Glucose	0,26	0,98	$1,02 \times 10^{-2}$	$1,06 \times 10^{-2}$

1) were obtained. The inoculum dose of bifidobacteria was 3,33 % v/v, and the initial concentrations of *Bac. cereus* was 0,33 % v/v. The cultivation was carried out for 10 h.

The study of the *Bif. adolescentis* growth in co-culture showed that the specific growth rate and metabolites yields considerably changed compared with pure culture. The specific growth rates of bifidobacteria in co-culture were higher in FOC and glucose too. It means that the non-probiotics stimulated the growth of the probiotics. Presumably, the bacilli have the strong protease complex [20] to enrich the nutrient medium with the peptides available for bifidobacteria.

The mass ratio of lactic and acetic acids during fermentation in a mixed culture changed markedly. Moreover, inFOS it was 1.5 times, and in glucose containing medium - about 3 times more for acetic acid. Also, the acid yields in relation to the accumulation of CFU of bifidobacteria in all cases were significantly lower in mixed cultures. (table 1).

The behavior of non-probiotics in co-culture was the most remarkable. The significant decreasing in the final content of bacilli in mixed culture compared with its count in pure culture was established for all variants of the experiments (Fig. 1). The initial growth rate of *Bac. cereus* in co-culture in FOS was higher than in pure culture. In all cases the growth of bacilli stopped after 6 hours from inoculation.

As a result of flow fermentation of probiotic and non-probiotic with the addition of oligofructose (prebiotic) and glucose (control), bacteria growth curves, concentration of metabolic products, consumption of non-prebiotic substrate were obtained, shown in figures 2 and 3.

Analyzing the growth curve of bifidobacteria on glucose (Fig. 2) showed the competition for a carbohydrate substrate, as a result of which the amount of the probiotic culture begins to fall.The specific growth rates of *Bac. cereus*were calculated: for pure culture – 0,61 h^{-1}, for mixed with bifidobacteria culture – 0,32 h^{-1}. The maximum concentration of acetic acid (7.1 g/L) is reached at 50 hours of cultivation, then it is washed out of the system, which correlates with a decrease in quantity of *Bif. adolescentis*. However,

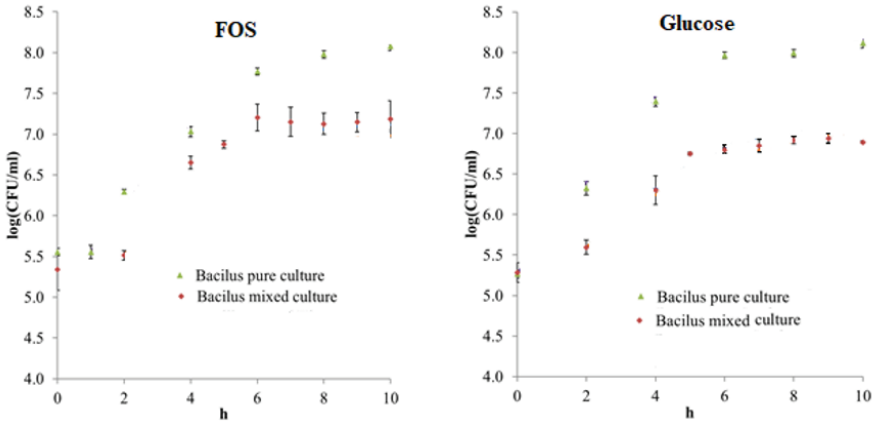

Figure 1: *Bac. cereus* growth curves in pure and co-culture in medium with FOS (left) or glucose (right). Each value of log10 of bacterial count is average from 3 measurements (p = 0.05).

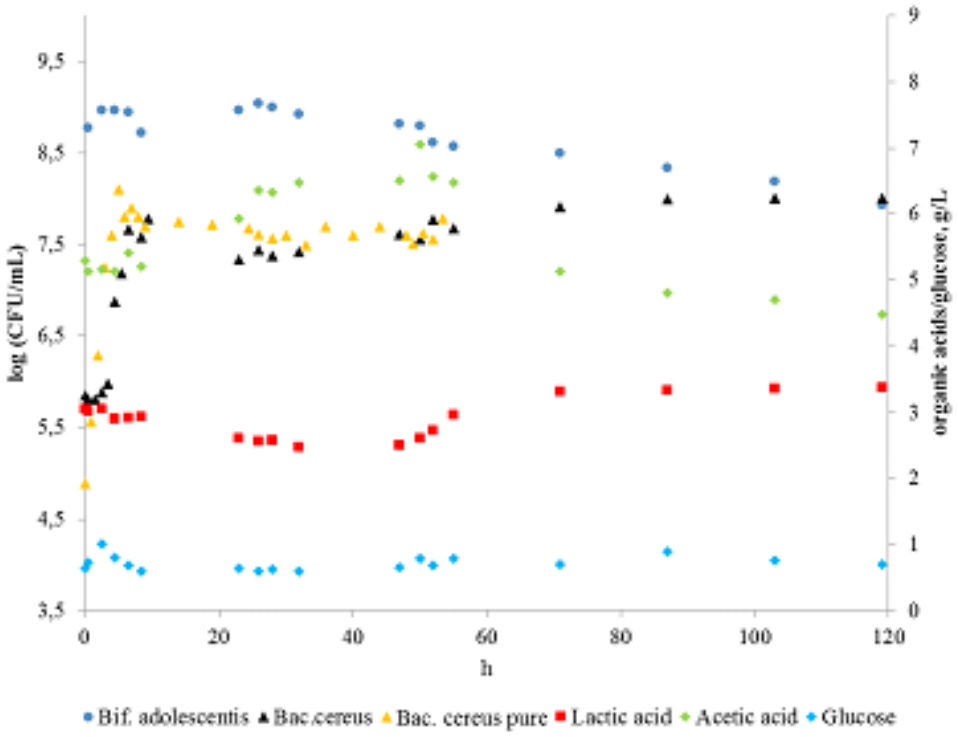

Figure 2: Dynamics of the population of *Bif. adolescentis* and *Bac. cereus*, the concentration of the substrate and metabolic products (lactic and acetic acids) during co-cultivation in comparison with the dynamics of the number of pure culture *Bac. cereus* on glucose (10 g/L) (non-prebiotic substrate).

there is a slight increase in the concentration of lactic acid. This can be explained by the change over the metabolism of bacilli from the consumption of glucose to the protein components of the medium under anaerobic conditions, in which they can produce lactate [21]. The experimental results show that bacilli suppress the growth of bifidobacteriain a medium with glucose due to their more active consumption of the carbohydrate substrate.

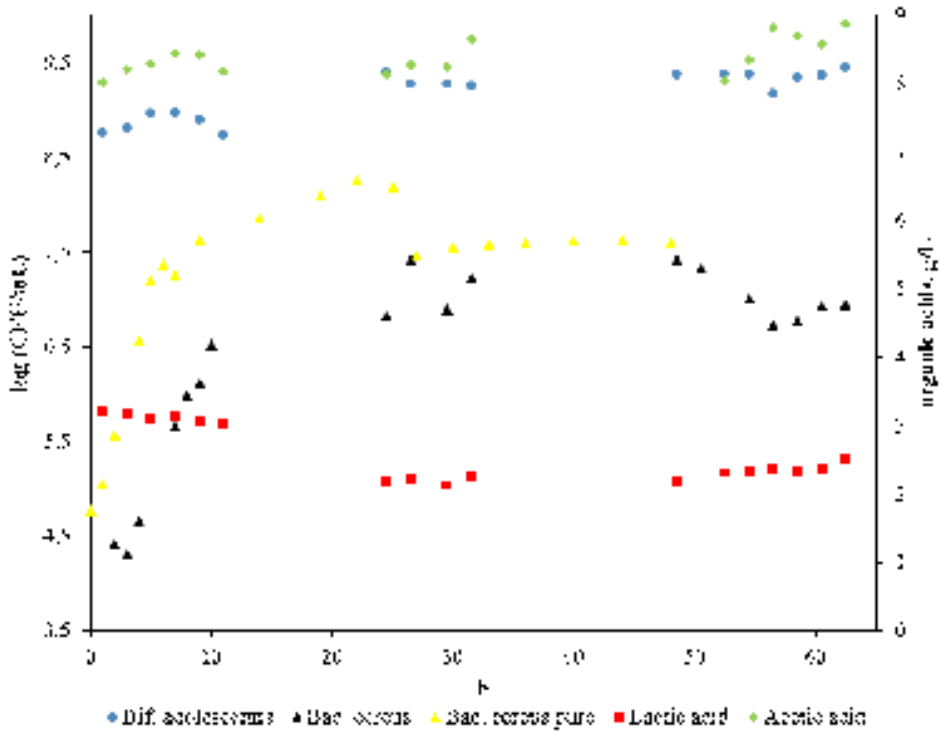

Figure 3: Dynamics of the amount of the colony forming units of *Bif. adolescentis* and *Bac. cereus*, the concentration of the substrate and metabolic products (lactic and acetic acids) during co-cultivation in comparison with the dynamics of the number of pure culture *Bac. cereus* on FOS (10 g/L) (prebiotic substrate).

Growth dynamics of Bac. cereuson a prebiotic substrate (Fig. 3) shows a lower growth rate in the exponential phase compared to the growth of a pure culture in both FOS and glucose containing medium. After 10 hours of cultivation together with bifidobacteria, the bacillus culture reaches dynamic balance. The maximum number of bacilli does not exceed the value for a pure culture, in contrast to cultivation on glucose. The count*Bif. adolescentis* and the concentration of acids on the FOS remain unchanged within the measurement error, which demonstrates a longer stay of the culture in a stable state of dynamic equilibrium compared to the experiment on glucose.

Growth parameters during co-cultivation on a prebiotic substrate show that the probiotic suppresses the growth of the contaminant from the initial moment of infection. To study the characteristics of the behavior of a probiotic, it is necessary to conduct longer experiments of the cultivation of bifidobacteria.

A series of experiments was carried out with varying the concentration of the prebiotic substrate, which were 2, 5, 10, 15 и 20 g/L (Fig. 4). The state of dynamic balance was reached within 6-10 hours (depending on the concentration of the substrate). In some experiments, the duration of studying the system in a state of dynamic balance ranged from 68 to 103 hours. It was found that the system remained stable during this

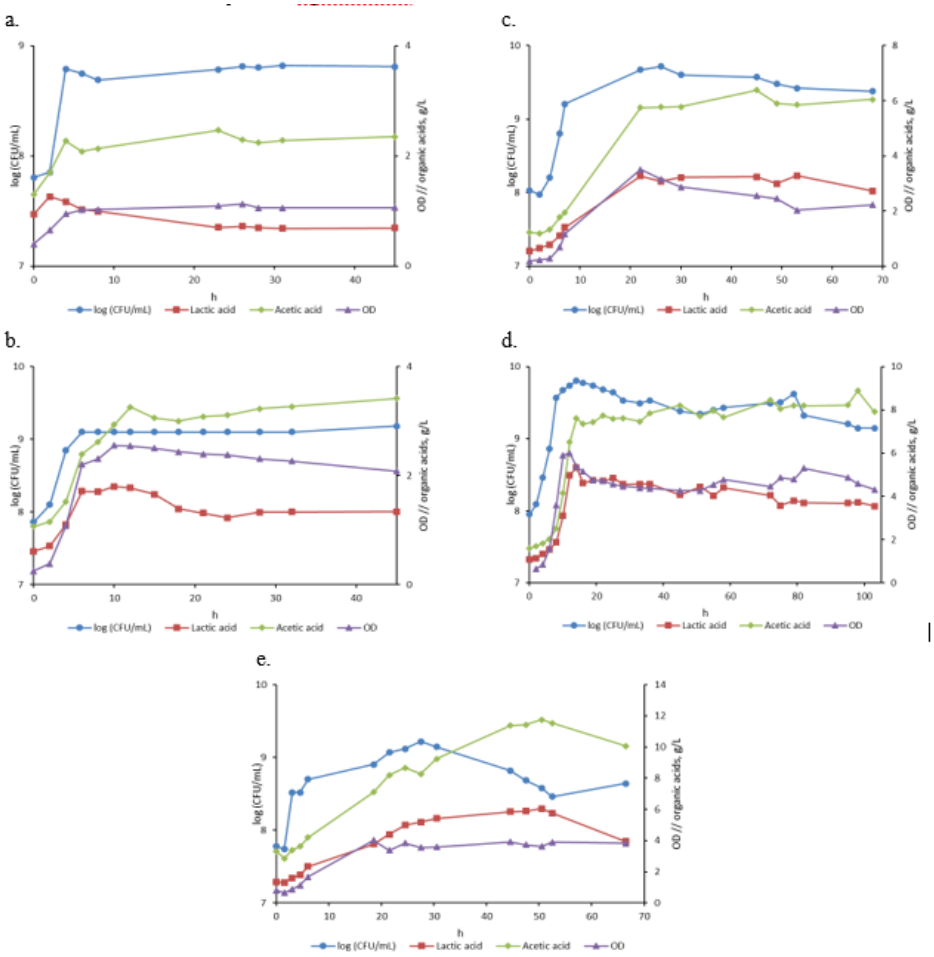

Figure 4: Dynamics of the amount of the colony forming units of *Bif. adolescentis*, opticaldensityand the concentration of metabolites (lactic and acetic acids) during flow fermentation at a concentration of FOS:a - 2 g/L, b – 5 g/L, c – 10 g/L, d – 15 g/L, e – 20 g/L.

time. Then, selectively, a bacillus culture was inoculated to the system with a substrate concentration of 5, 10, and 15 g/L for co-cultivation (Fig. 5). A relationship between the number of bifidobacteria and the concentrations of metabolites, as well as with a delay in the growth and number of bacilli cells in a state of dynamic equilibriumis observed.

4. Conclusion

The main regularities of growth of pure and mixed cultures of *Bifidobacteriumadolescentis* ATCC 15703 and *Bacillus cereus* ATCC 9634 in media with prebiotic (oligofructose) and glucose under continuous of flow cultivation in conditions close to the human large intestine and under conditions of batch fermentation have been established.It has been shown that in co-cultivation, the growth of bacilli is suppressed due to the release of inhibitory metabolites (lactic and acetic acids) by the probiotic in both cases.In the

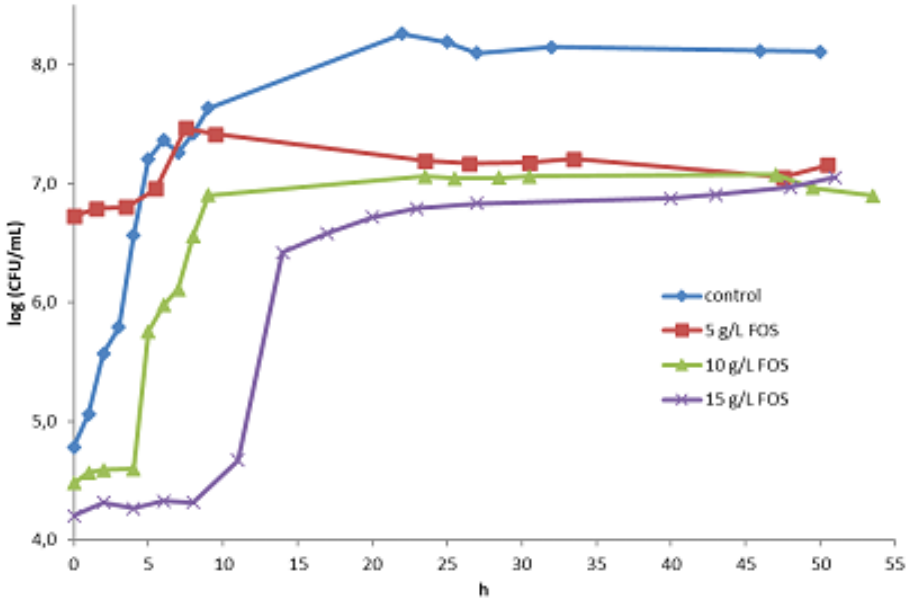

Figure 5: Dynamics of the amount of the colony forming units of bacilli in a mixed culture with *Bif. adolescentis*at various concentrations of the substrate. The culture of bifidobacteria was kept in a state of dynamic balance for at least 30 hours prior to inoculate the bacilli. Control is pure culture of *Bacilluscereus*.

case of batch fermentation, the inhibitory effect is higher, since it includes the action of hydrogen ions, which is excluded during flow fermentation with constant pH.During flow fermentation, increasing the FOS concentration increase the lag-phase of bacilli and decrease the count at the moment of reaching dynamic balance, although their specific growth rate does not change significantly.Thus, the study of synbiotic compositions in the context of the suppression of various pathogens or food contaminants is a promising direction of scientific redearch in the prevention and control of various intestinal bacterial infections.

5. Acknowledgement

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