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Antagonistic activity of synbiotics: Response surface modeling of various factors

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Abstract:
Synbiotic compositions have a great potential for curing microbial intestinal infections. Novel targeted synbiotics are a promising field of the modern functional food industry. The present research assessed the effect of various fructan fractions, initial probiotic counts, and test strains on the antagonistic properties of synbiotics.

The research involved powdered roots of *Arctium lappa* L. and strains of *Bifidobacterium bifidum*, *Bacillus cereus*, and *Salmonella enterica*. The exp eriment was based on the central composite rotatable design. A water extract of *A. lappa* roots was purified and concentrated. Fructan fractions were precipitated at various concentrations of ethanol, dried, and subjected to carbon-13 nuclear magnetic resonance (13C-NMR) spectrometry. The bifidobacteria and the test strains were co-cultivated in the same medium that contained one of the fractions. Co-cultivation lasted during 10 h under the same conditions. The acid concentrations were determined by high-performance liquid chromatography to define the synbiotic factor.

The obtained fructans were closer to commercial oligofructose in terms of the number and location of NMR peaks. However, they were between oligofructose and inulin in terms of signal intensity. The response surface analysis for bacilli showed that the minimal synbiotic factor value corresponded to the initial probiotic count of 7.69 log(CFU/mL) and the fructan fraction precipitated by 20% ethanol. The metabolites produced by the bacilli also affected their growth. The synbiotic factor response surface for the experiments with *Salmonella* transformed from parabolic to saddle shape as the initial test strain count increased. The minimal synbiotic factor value corresponded to the lowest precipitant concentration and the highest probiotic count.

The research established a quantitative relationship between the fractional composition of fructans and the antagonistic activity of the synbiotic composition with bifidobacteria. It also revealed how the ratio of probiotic and pathogen counts affects the antagonism. The proposed approach can be extrapolated on other prebiotics and microbial strains *in vivo*.

Keywords: Bifidobacteria, *Bacillus cereus*, *Salmonella enterica*, *Arctium lappa* L. fructans, synbiotics, antagonism, co-culture, rotatable central composite design, response surface methodology

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INTRODUCTION

Intestinal microbiota affects human health and vitality. Microbial community is a powerful and multifunctional metabolic system that modulates immunity, suppresses pathogens, and produces various vitamins [1, 2]. A disturbed qualitative and quantitative microbial composition leads to various alimentary and chronic diseases. For instance, low counts of *Bacteroides* and *Firmicutes*, if accompanied by excessive proteobacteria, fusobacteria, and the mucin-decomposing *Ruminococcus gnavus*, can trigger Crohn's disease, ulcerative colitis, obesity, and diabetes [3].

However, some intestinal microbes inhibit pathogens and food contaminants by producing such antimicrobial substances as organic acids and bacteriocins or competing for nutrients and adhesion sites [4–7]. If it were not for them, unwanted microorganisms would cause constant harm to human health by producing various toxins or enzymes. For instance, *Bacillus cereus* is a common

food contaminant that produces two types of toxins and causes vomiting and diarrhea intoxication [8]. *B. cereus* spores are resistant to heat treatment and chemical preservation [9].

Non-typhoid *Salmonella* is another wide-spread cause of foodborne diseases [10]. *Salmonella enterica* s. *Typhimurium* is often resistant to antibiotics and can develop biofilms, thus causing gastroenteritis, vomiting, and diarrhea [11]. Antibiotic-resistant bacteria are the most dangerous causes of intestinal infections [12]. Therefore, novel non-antibiotic ways to suppress these pathogens and food contaminants for therapy and prevention are one of the most urgent tasks of the modern medicine. Synbiotic compositions offer a potential solution to this problem because they are extremely effective in inhibiting the growth, activity, and pathogenesis of specific undesirable microorganisms.

Probiotics, prebiotics, and synbiotics are parts of functional foods that inhibit unwanted members of intestinal microbiota [13]. These food additives are known to increase α-diversity, combat obesity, improve immunity, and counteract pathogens [13–16]. Synbiotics are the most effective type because they possess synergistically enhanced beneficial properties of probiotics and prebiotics [17].

For synbiotics, the most important criteria are their inhibiting properties, adhesion to intestinal epithelial cells, and pathogen toxicity. Antagonistic research of synbiotic combinations is a promising strategy for developing new synbiotics. Ruiz *et al.* studied the combined antimicrobial activity of a synbiotic based on *Bifidobacterium longum* subsp. *infantis* and galactooligosaccharides against such enteric pathogens as *Escherichia coli*, *Cronobacter sakazakii*, *Listeria monocytogenes*, and *Clostridium difficile*. *C. sakazakii* and *C. difficile* proved to be the most effective pathogen inhibitors [18]. Co-cultivation of *B. longum* or *Bifidobacterium breve* with *C. difficile* in a medium with commercial fructooligosaccharides reduced the pathogen growth, as well as the toxicity of its metabolites [19].

Śliżewska and Chlebicz-Wójcik focused on the effect of various prebiotics co-cultivated with lactobacilli on pathogenic *S. enterica* of various serovars and *L. monocytogenes*. Inulin demonstrated the greatest antagonistic activity, although the effect depended on the test strain [20]. Obviously, the effectiveness of one and the same composition depends on the pathogen. The inhibitory effect can be measured by the inhibitory metabolites produced by probiotics. This effect can be expressed in terms of inhibition constants (K_i) or minimal inhibitory concentrations. The synbiotic factor is another quantitative criterion for evaluating the effectiveness of synbiotic compositions. It shows how many times the specific growth rate of a pathogen or microbial contaminant decreases under the action of acids produced by a probiotic when they are co-cultivated in the same medium with this prebiotic [21].

Plant extracts are common sources of prebiotic substances. In addition to polysaccharides of various molecular weights, they may contain non-carbohydrate substances with a potential beneficial effect, e.g., polyphenols [22, 23]. Precipitation with different concentrations of ethanol can separate plant carbohydrates into fractions with different degrees of polymerization. Polysaccharides with a higher degree of polymerization require a lower concentration of ethanol. As the alcohol concentration increases, the average degree of polymerization of the precipitated fraction decreases [24, 25]. Polysaccharides with a high degree of polymerization are not metabolized by pathogens without extracellular hydrolases. However, they can be metabolized by many types of probiotics, e.g., bifidobacteria and some lactobacilli, which determines their significant prebiotic potential [26]. In our previous research, we evaluated the effectiveness of a synbiotic composition *in vitro* by the degree of its antagonism against staphylococci. It depended on the fractional composition of *Arctium lappa* fructans, as well as on the ratio of the initial probiotic and pathogen counts [27].

The response surface methodology was developed by Box and Wilson [28]. It is a powerful tool for establishing quantitative relationships between various factors and the response function, also by taking into account the mutual effect of factors in multiparameter equations. Shuhaimi *et al.* used this method to optimize the composition of a synbiotic that consisted of *Bifidobacterium pseudocatenulatum* and several prebiotics, while Pandey and Mishra tested this method on a soy drink with lactic acid bacteria and organophosphates [29, 30].

Few researchers venture beyond simple optimization to look for the patterns between various factors and the response function. This approach proved quite effective in studying the change patterns in microbial communities under various environmental factors [31, 32]. Antagonism is a type of relationships in microbial communities. Our research objective was to use the response surface method to evaluate the effect of fructan fractional composition, the initial counts of probiotics and the pathogen test strain on the antagonism of the synbiotic against *B. cereus* and *S. enterica*.

STUDY OBJECTS AND METHODS

Plant raw materials and obtaining fructan fractions. To isolate fructans, we used burdock root powder (*Arctium lappa* L.) in accordance with pharmacopeial monograph 2.5.0025.15 of the Russian Pharmacopoeia. The powder was diluted with distilled water in a ratio of 1:12 (g dry solids per 1 mL extractant) and extracted twice at 75°C and pH 6.5 for 30 min with constant stirring. The pulp was separated by vacuum filtration. To separate high-molecular impurities, the extract was ultrafiltered at 45°C through a hollow fiber module (AR-0.5-20PS, NPO Biotest, Kirishi, Russia) with a retention threshold of 20 kDa. The permeate was stirred with active clarifying carbon at a rate of

15 g/L for 30 min until the extract became colorless. The activated charcoal was separated by vacuum filtration [33].

The extract was evaporated using a rotary film evaporator (model 561-01110-00 with glass set G1, Heidolph, Germany) at 45°C until the carbohydrate concentration reached 170–200 g/L. To separate the carbohydrates into fractions with different degrees of polymerization, the extract was precipitated with varying ethanol concentrations (20.0, 32.2, 50.0, 67.8, and 80.0%) at 4°C for 4 days [24].

The precipitates were separated by centrifugation at 5000 rpm for 15 min and dried in a ScanVac Coolsafe 100-9 freeze-dryer under the following temperature and time conditions: 0°C – 8 h, 5°C – 8 h, 10°C – 6 h, 15°C – 6 h, and 20°C – 6 h. The samples were diluted 1:1 with a 10% solution of trichloroacetic acid and hydrolized for 40 min in a boiling water bath. After that, the content of fructans was determined by the modified Bertrand method.

Microbial objects and cultivation conditions. All the bacterial cultures were obtained from the National Bioresource Center of the All-Russian Collection of Industrial Microorganisms in the National Research Center of Kurchatov Institute (VKPM). *Bifidobacterium bifidum* (AS-1666, ATCC 29521^T) served as a probiotic culture. *Bacillus cereus* (B-8076, ATCC 9634) was used as a model food contaminant. *Salmonella enterica* (B-5300) was a model intestinal pathogen. The medium described in [34] was modified to obtain inoculums and co-cultivate the probiotic and test strains.

The composition of the carbohydrate-free medium was as follows (g/L): casein trypton (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, and L-cysteine – 0.5 (pH 7.0). All the components were dissolved in 80% of the required amount of distilled water and autoclaved at 115°C for 30 min. The fructan precipitates were dissolved in distilled water (20% of the required medium volume) and sterilized separately under the same conditions. Prior to inoculation, carbohydrates were added to the medium aseptically until their concentration was 8 g/L.

Inoculums were cultivated at 37°C and stirred at 180 rpm under anaerobic conditions (2% CO_2, 98% N_2) in a CB-210 CO_2 incubator (Binder, Germany) for 12 h without maintaining a constant pH. After that, the inoculums were centrifuged at 6000 rpm and 4°C for 2 min and washed twice in sterile saline (9 g/L NaCl). Then the precipitate was resuspended in a carbohydrate-free medium to obtain suspensions with an optical density depending on the bacterial count. To achieve the selected initial count of the probiotic and the test strain, 0.5 mL of the obtained solution was added to the media with pre-added fructans. To determine the synbiotic factor, co-cultivation lasted during 10 h under the same conditions. Sampling took place at the beginning and end of fermentation.

Microbial count. Microbial count was conducted in triplicate by seeding tenfold dilutions in Petri dishes with the media. Colonies of *B. cereus* and *S. enterica* were counted after 24 h of aerobic growth at 37°C in MRS medium [35]. *B. bifidum* colonies were counted after 48 h of growth in BFM medium with the following composition (g/L): peptone – 10, NaCl – 5.0, lactulose – 5.0, L-cysteine – 0.5, riboflavin – 0.01, yeast extract – 7, meat extract – 5, starch – 2, thiamine chloride – 0.01, and lithium citrate – 3.3 [36]. The pH was adjusted to 5.5 by adding propionic acid (5 mL/L). The dishes were incubated under anaerobic conditions at 37°C using a BD GasPak™ Anaerobic Container System.

Determining the content of organic acids. The concentration of organic lactic and acetic acids was determined by high-performance liquid chromatography (HPLC) according to a slightly modified standard procedure by the refractometric signal [37]. The experiment involved an Agilent 1220 Infinity chromatograph (Santa Clara, CA, USA) with an Agilent Hi-Plex H column (250×4.6 mm). The supernatant was centrifuged at 12 000 rpm for 15 min, then filtered through 0.45-μm cellulose acetate membranes (HAWP, MF-Millipore, St. Louis, MO, USA). Other parameters included: sample volume – 3 μL, temperature – 50°C, mobile phase flow rate (0.002 M H_2SO_4) – 0.3 mL/min. To prepare calibration solutions, the concentrated organic acids were diluted in their mobile phase to concentrations of 1, 5, and 10 g/L.

Determining the structure of fructans. The structure of the isolated fructans was analyzed using carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrometry following the procedure described by Mariano *et al.* [38]. One-dimensional spectra were obtained at 298 K on a BRUKER CXP-200 NMR spectrometer (50.3 MHz) (Bruker, Germany) in an aqueous solution of D_2O. Inulin (Orafti ® HSI, BENEO-ORAFTI, Belgium) and oligofructose (Orafti ® P95, BENEO-ORAFTI, Belgium) served as standard.

Calculating the synbiotic factor. The synbiotic factor was calculated in accordance with the previously approach proposed by Karetkin *et al.* and Evdokimiova *et al.* [21, 27]. The microbial count, pH, and the concentration of organic acids were determined at the initial and final stages of co-cultivation. Based on the data obtained, the synbiotic factor was calculated as follows:

$$SF = \frac{pH - pH_{min}}{pH_{opt} - pH_{min}} \times \left[1 - \left(\frac{[LA]}{MIC_{LA}}\right)^{\alpha}\right] \times \left[1 - \left(\frac{[AA]}{MIC_{AA}}\right)^{\beta}\right] \quad (1)$$

where SF is the synbiotic factor; pH_{opt} is pH optimal for test strain growth; pH_{min} is pH the minimal for test strain growth; [LA] is the concentration of undissociated lactic acid, (mg/mL); [AA] is the concentration of undissociated acetic acid, mg/mL; MIC_{LA} is the minimal inhibiting concentration of lactic acid, mg/mL; MIC_{AA} is the minimal inhibiting concentration of acetic acid,

Table 1 Minimal inhibitory concentrations, constants, and optimal and minimal pH during the process of *Bacillus cereus* or *Salmonella enterica* inhibition by lactic and acetic acids

Test strain	pH_{opt}	pH_{min}	MIC_{LA}, mg/mL	MIC_{AA}, mg/mL	α	β
Bacillus cereus	7.0	4.9	3.48	3.20	0.25	0.40
Salmonella enterica	7.0	5.0	2.25	1.77	1.70	0.90

mg/mL; α and β are constants for *B. cereus* or *S. enterica*, which we defined in [39] (Table 1).

Design of experiment and statistical analysis. The central composition rotatable design was applied to study the effect of the following parameters on the co-cultivation: the precipitant concentration x_1, the fractional composition of *A. lappa* fructans, the initial count (decimal logarithm) of bifidobacteria (x_2), and test strain cells (x_3). Synbiotic factor (Y_1) and final test strain count (Y_2) were chosen as response functions. The variation levels were determined based on data obtained from [21, 27] (Tables 3 and 4). The response function was presented as follows:

$$Y_k(x_1, x_2, x_3) = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + \quad (2)$$
$$+ b_{23}x_2x_3 + b_{13}x_1x_3 + b_{123}x_1x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

The significance test of the coefficients for Eq. (2) was based on the t-test. The adequacy of the equation was assessed by the Fisher criterion at P = 0.05. Response surfaces were calculated and constructed using the MathLab software. The scanning method with a variable step as in [40] was applied to determine the extreme values of the factors. The method consists in a sequential search for points in the parametric space using the GeoGebra Classic software 6.0.694.0 (University of Salzburg, Salzburg, Salzburg state, Austria).

RESULTS AND DISCUSSION

^{13}C-NMR specters of *Arctium lappa* L. root fructan fractions. Figure 1 illustrates ^{13}C-NMR specters of standard inulin and oligofructose, purified from *A. lappa* L. fructan fractions and precipitated by different concentrations of ethanol.

The analysis was based on the difference between the chemical shifts of the carbon atoms of the monomers located inside the chain of oligo- and polysaccharides and the atoms of the terminal monomers [24]. The chemical shifts of carbon atoms in the standard and test samples are typical of inulin-type fructans (Table 2). The obtained spectra of fructan fractions were closer to those of commercial oligofructose in terms of the number and location of peaks. In terms of signal intensity, they were between standard oligofructose and highly purified inulin. None of the test samples demonstrated peaks at the terminal C-2 atom of D-fructofuranose. However, the test samples showed an increase in the relative areas of the peaks, as well as an increase in the precipitant concentration for all carbon atoms of the D-fructofuranose residues within the chain (forming a 2→1 bond).

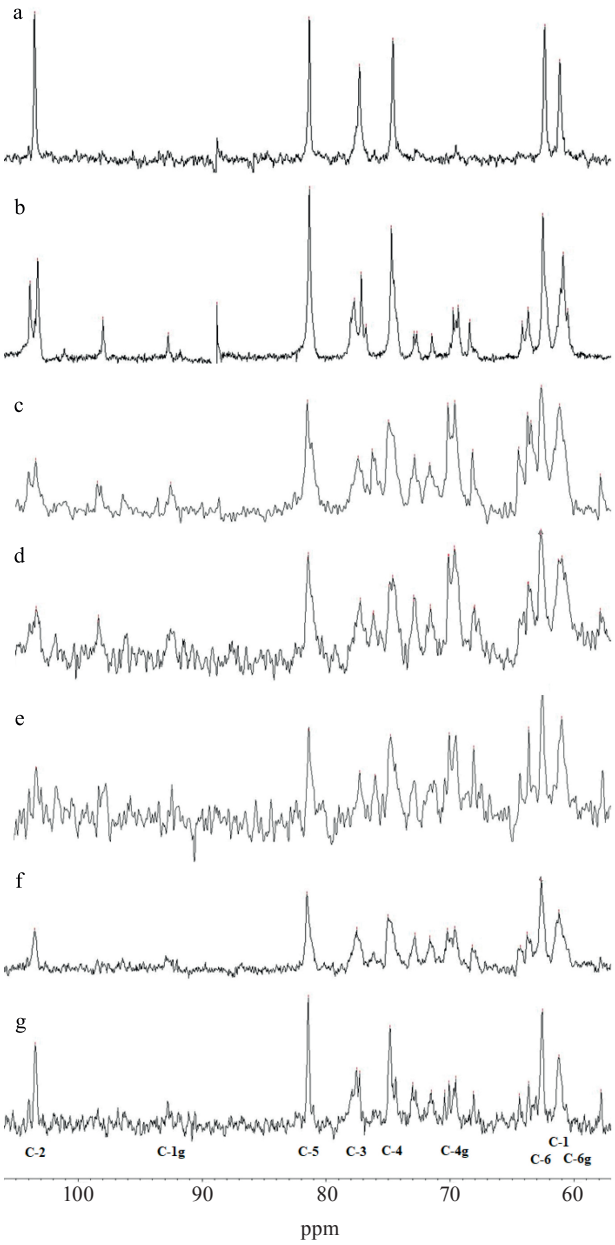


Figure 1 ^{13}C-NMR specters in distilled water with D_2O and (a) HSI inulin, (b) oligofructose and *Arctium lappa* L. fructan fractions precipitated by ethanol with concentrations, (c) 20.0% (Burd-20), (d) 32.2% (Burd-32), (e) 50.0% (Burd-50), (f) 67.8% (Burd-68), and (g) 80.0% (Burd-80)

All the peak areas for the corresponding carbon atoms were smaller than for inulin, and the values obtained for Burd-50 and Burd-68 were closest to

Table 2 ^{13}C-NMR chemical shifts of *β*-D-fructofuranose and *α*-D-glucopyranose of HSI inulin standard samples, oligofructose, and experimental samples of *Arctium lappa* L. root fructan fractions precipitated with various concentrations of ethanol: 20% (Burd-20), 32.2% (Burd-32), 50% (Burd-50), 67.8% (Burd-68), and 80% (Burd-80)

Carbon atom	Chemical shift, ppm						
	Inulin	Oligofuctose	Burd-20	Burd-32	Burd-50	Burd-68	Burd-80
C-2 f (terminal)	–	103.88	–	–	–	–	–
C-2 f (2→1 bond)	103.4262	103.2645	104.26	104.29	103.40	103.43	103.43
	–	97.98	99.31	99.25	–	–	–
C-1 g (terminal)	–	92.7261	93.41	–	–	–	–
	–	88.76	–	–	–	–	–
C-5 f (terminal)	–	–	–	–	–	–	–
C-5 f (2→1 bond)	81.3253	81.3253	82.40	82.30	81.44	81.50	81.44
	–	77.71	–	–	–	–	–
C-3 f (2→1 bond)	77.2824	77.1477	78.31	78.06	77.32	77.48	77.54
C-3 f (terminal)	–	76.77	77.16	77.00	76.07	–	77.32
	–	–	–	–	–	–	–
C-4 f (2→1 bond)	74.5872	74.70	75.85	75.76	74.83	74.96	74.86
C-4 f (terminal)	–	–	–	75.44	–	–	–
C-3 g (terminal)	–	72.8892	73.75	73.75	–	72.79	73.04
C-5 g (terminal)	–	72.6736	–	–	–	–	–
C-2 g (terminal)	–	71.4337	72.53	72.41	–	71.58	71.54
C-4 g (terminal)	69.4	69.3045	71.06	70.94	70.11	70.17	70.46
	–	68.39	69.09	68.83	68.13	68.19	68.13
C-6 f (2→1 bond)	–	64.1566	65.38	64.49	64.30	64.30	64.43
C-6 f (terminal)	–	63.67	64.65	63.47	63.69	63.79	63.69
	–	–	64.36	–	–	–	–
C-1 f (2→1 bond)	62.3777	62.4858	63.53	62.06	62.61	62.61	62.57
C-1 f (terminal)	61.16	60.84	62.06	61.78	61.04	61.20	61.27
C-6 g (terminal)	–	60.49	58.78	58.71	57.88	–	57.85

oligofructose. The differences in the relative proportions of peak areas for Burd-20 and Burd-32 were small and manifested as unidentified peaks in the Burd-20. Probably, carbohydrates of similar molecular weight were precipitated at these ethanol concentrations. No correlation was observed between the relative proportions of the peak areas for the terminal atoms of glucopyranose and fructofuranose.

Synbiotic antagonism to *Bacillus cereus* and response surface analysis. To assess the effect of various factors on the anti-*B. cereus* activity of the synbiotic composition, the experiment was carried out according to a central composition rotatable design. The limiting values of ethanol concentration were chosen as 20 and 80% as in [27]. The average polymerization degree of the precipitated carbohydrate fraction was at its highest at 20% of ethanol.

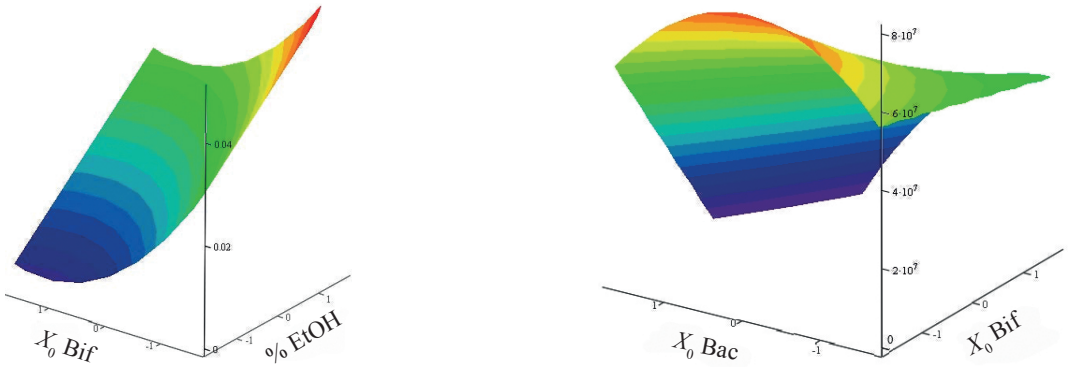
Zeaiter *et al.* used 33% ethanol to obtain a fraction of inulin-type artichoke fructans with an average degree of 32–42 [26]. Table 3 demonstrates the planning matrix, as well as the experimental and calculated values of response functions, i.e., the synbiotic factor and the final test strain cell count.

The coefficients of the response function equation were determined from the values of the synbiotic factor and the final bacterial count. The response surface was constructed according to Eq. (2) (Fig. 2). The adequacy of the equations was confirmed by Fisher's criterion $F = 1.681$ and 1.66: it was below the tabulated $F = 4.704$ at $P = 0.05$.

$$Y_1 = 0.0211 + 0.008x_1 - 0.0074x_2 + 0.0032x_2^2 \quad (3)$$

$$Y_2 = 4.9 \times 10^7 - 1.6 \times 10^7 x_2 - 1.2 \times 10^7 x_3 - \\ - 7.1 \times 10^6 x_2 x_3 - 7.4 \times 10^6 x_3^2 \quad (4)$$

The synbiotic factor reduced of the specific growth rate of the test strain. It showed how many times the specific growth rate decreased relative to the optimal value under the effect of inhibitors produced by the probiotic and the prebiotic. The maximal inhibition corresponded to the lowest value of the synbiotic factor [21]. The synbiotic factor of the composition of *Bifidobacterium bifidum* and *A. lappa* root fructans had a positive linear dependence on the precipitant concentration (x_1). Therefore, the composition with fructans precipitated by the lowest alcohol concentration had the greatest inhibitory effect on *B. cereus* because it had the highest average degree of polymerization. This result confirms the data obtained by us before [27]. The dependence of the synbiotic factor on the initial probiotic count (x_2) was parabolic and reached its minimum at +1.156, which corresponded



a b

Figure 2 Synbiotic factor response surface (a) and final bacterial count (b), CFU/mL

Table 3 Range of variation and encoding of variables: experimental and calculated values of response functions for *Bacillus cereus*

Test No.	Factors						Synbiotic factor		Final bacterial count, lg(CFU/mL)*	
	Precipitant concentration (EtOH), %		Initial prebiotic count, lg(CFU/mL)		Initial bacterial count, lg(CFU/mL)					
	z_1	x_1	z_2	x_2	z_3	x_3	SF_{obs}	SF_{pred}	$X_{bac\ obs}$	$X_{bac\ pred}$
1	67.8	+1	7.6	+1	6.4	+1	0.0267	0.0249	5.72	6.77
2	67.8	+1	7.6	+1	4.6	–1	0.0310	0.0249	7.48	7.65
3	67.8	+1	6.4	–1	6.4	+1	0.0420	0.0397	7.80	7.72
4	67.8	+1	6.4	–1	4.6	–1	0.0433	0.0397	7.77	7.80
5	32.2	–1	7.6	+1	6.4	+1	0.0136	0.0089	5.43	6.77
6	32.2	–1	7.6	+1	4.6	–1	0.0092	0.0089	7.58	7.65
7	32.2	–1	6.4	–1	6.4	+1	0.0224	0.0236	7.66	7.72
8	32.2	–1	6.4	–1	4.6	–1	0.0244	0.0236	7.79	7.80
9	20.0	–1.682	7.0	0	5.5	0	0.0188	0.0076	7.77	7.69
10	80.0	+1.682	7.0	0	5.5	0	0.0404	0.0346	7.63	7.69
11	50.0	0	6.0	–1.682	5.5	0	0.0471	0.0425	7.82	7.88
12	50.0	0	8.0	+1.682	5.5	0	0.0179	0.0177	7.51	7.34
13	50.0	0	7.0	0	4.0	–1.682	0.0268	0.0211	7.77	7.69
14	50.0	0	7.0	0	7.0	+1.682	0.0301	0.0211	6.63	6.84
15	50.0	0	7.0	0	5.5	0	0.0213	0.0211	7.74	7.69
16	50.0	0	7.0	0	5.5	0	0.0122	0.0211	7.68	7.69
17	50.0	0	7.0	0	5.5	0	0.0239	0.0211	7.76	7.69
18	50.0	0	7.0	0	5.5	0	0.0241	0.0211	7.70	7.69
19	50.0	0	7.0	0	5.5	0	0.0205	0.0211	7.56	7.69
20	50.0	0	7.0	0	5.5	0	0.0237	0.0211	7.66	7.69

* the response function was calculated as CFU/mL; the results are given on a logarithmic scale

to 7.69 lg(CFU/mL). As x_2 rose (> +1.156), the synbiotic factor also increased.

All experimental values appeared to be much higher than those obtained by calculation, both at the minimal point and at high values of x_2. Apparently, the observed decrease in the antagonistic activity could be ignored. All the coefficients at x_3 proved insignificant, and the initial test strain count did not affect the synbiotic factor. Within the range of the variables, the minimal value of the synbiotic factor (maximal suppression of the test strain) was 0.0033 and lied at the point with coordinates –1.682 and 1.156, which corresponded to the initial probiotic count of 7.69 lg(CFU/ml) and the *A. lappa* fructan fraction precipitated with 20% ethanol.

As the initial bifidobacterial count (x_2) increased, the final bacterial count decreased (Fig. 2b). The dependence of the final test strain count (Y_2) on the initial one (x_3) was parabolic. The maximal value of the response function was reached when the bifidobacterial count was minimal, i.e., 6.0 lg(CFU/mL), and the initial test strain count in the design center was 5.5 lg(CFU/mL). At these values, the inhibition was least effective. The minimal final test strain count was around the highest seed doses of both the probiotic and

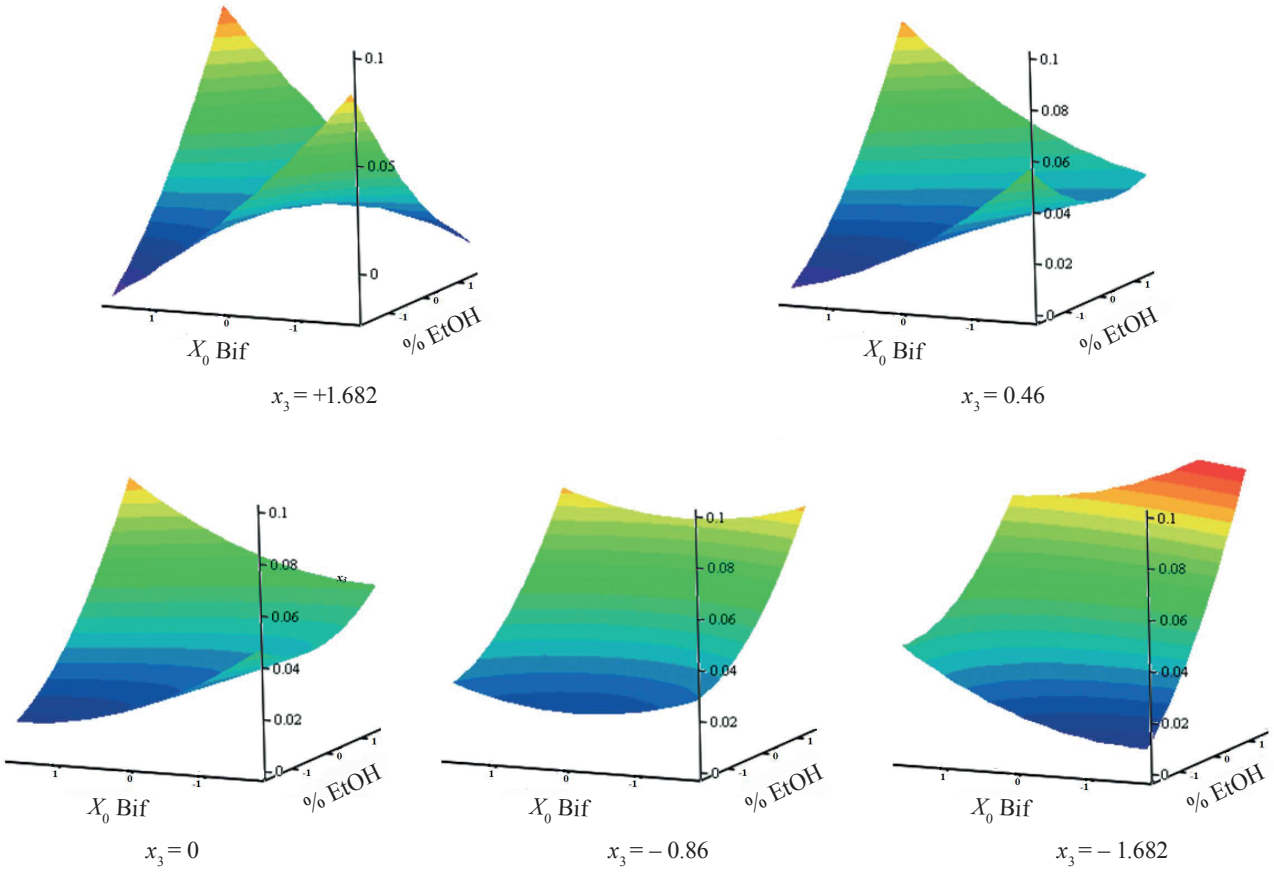


Figure 3 Synbiotic factor response surface as a function of ethanol concentration (x_1) and initial probiotic count (x_2) at fixed initial *Salmonella* count (x_3)

the test strain. As the initial probiotic and test strain concentrations increased, the final bacterial count plummeted. Probably, bacilli inhibited their own growth by their own metabolites, i.e., lactic acid.

Antagonism of synbiotic compositions against *Salmonella enterica* and response surface analysis. Table 4 shows the design matrix with experimental and calculated values of the response functions for *S. enterica*. The variation range of variables in natural coordinates did not differ from that of bacilli, except for the shift in the initial test strain count by +1 lg(CFU/mL).

The response surface analysis for synbiotic factor (Y_1) was represented as the following equation confirmed by Fisher's criterion ($F = 3.99 < 4.87$, $P = 0.05$):

$$Y_1 = 0.029 + 0.012x_1 - 0.007x_3 + 0.006x_1x_2 - 0.006x_1x_3 + \\ + 0.007x_1x_2x_3 + 0.004x_1^2 + 0.003x_2^2 \quad (5)$$

The coefficients for all factors and their pairwise interactions turned out to be significant. The response surfaces were calculated for fixed (Fig. 3). For all the surfaces obtained, the smallest value of the response function within the variation range was obtained when the precipitant concentration was minimal. When was below 0.46, which corresponded to the initial *Salmonella* count (6.91 lg(CFU/mL)), the response surface was parabolic, and its analytical minimum was outside the variation range. These surfaces demonstrated an increase in the initial bifidobacterial count, which followed the increase in the initial *Salmonella* count.

The larger bifidobacterial count resulted in the greatest suppression, which varied from $x_3 = -1.682$ to $x_3 = 0$. Thus, the response surface method made it possible to define the critical value of the *Salmonella* count (6.5 lg(CFU/mL)). When this value was exceeded, only the maximal count of viable bifidobacterial cells could inhibit the pathogen. If the initial pathogen count exceeded 6.91 lg(CFU/mL), the response surfaces had a saddle shape.

The global minimum of the response function within the variation range was determined by the variable step scanning method. Initially, all variables for each coordinate had an interval with two equal subintervals. The values of the function were calculated at the nodes of the resulting grid to select the optimal point with the lowest synbiotic factor. Subsequently, the interval was cut in two. The calculation cycles continued until the interval along one of the coordinates fell below 0.001. The minimum was determined at the border of the region in coordinates –1.682, +1.682, and +1.682. Therefore, the greatest antagonistic effect was expected at the lowest alcohol concentration of 20% and the

Table 4 Range of variation and encoding of variables: experimental and calculated values of response functions for *Salmonella enterica*

Test No.	Factors						Synbiotic factor		Final bacterial count, lg(CFU/mL)*	
	Precipitant concentration (EtOH), %		Initial prebiotic count, lg(CFU/mL)		Initial bacterial count, lg(CFU/mL)					
	z_1	x_1	z_2	x_2	z_3	x_3	SF_{obs}	SF_{pred}	$X_{sal\ obs}$	$X_{sal\ pred}$
1	67.8	+1	7.6	+1	7.4	+1	0.0544	0.0477	8.55	8.58
2	67.8	+1	7.6	+1	5.6	–1	0.0636	0.0596	8.53	8.47
3	67.8	+1	6.4	–1	7.4	+1	0.0233	0.0214	8.71	8.67
4	67.8	+1	6.4	–1	5.6	–1	0.0689	0.0616	8.64	8.59
5	32.2	–1	7.6	+1	7.4	+1	0.0114	0.0088	8.58	8.58
6	32.2	–1	7.6	+1	5.6	–1	0.0265	0.0267	8.41	8.47
7	32.2	–1	6.4	–1	7.4	+1	0.0328	0.0351	8.60	8.67
8	32.2	–1	6.4	–1	5.6	–1	0.0278	0.0247	8.60	8.59
9	20.0	–1.682	7.0	0	6.5	0	0.0225	0.0202	8.51	8.49
10	80.0	+1.682	7.0	0	6.5	0	0.0525	0.0602	8.47	8.49
11	50.0	0	6.0	–1.682	6.5	0	0.0468	0.0371	8.61	8.64
12	50.0	0	8.0	+1.682	6.5	0	0.0221	0.0371	8.46	8.44
13	50.0	0	7.0	0	5.0	–1.682	0.0399	0.0413	8.59	8.63
14	50.0	0	7.0	0	8.0	+1.682	0.0179	0.0162	8.77	8.74
15	50.0	0	7.0	0	6.5	0	0.0328	0.0287	8.61	8.62
16	50.0	0	7.0	0	6.5	0	0.0289	0.0287	8.61	8.62
17	50.0	0	7.0	0	6.5	0	0.0334	0.0287	8.65	8.62
18	50.0	0	7.0	0	6.5	0	0.0244	0.0287	8.63	8.62
19	50.0	0	7.0	0	6.5	0	0.0230	0.0287	8.63	8.62
20	50.0	0	7.0	0	6.5	0	0.0308	0.0287	8.57	8.62

* the response function was calculated as CFU/mL; the results are given on a logarithmic scale

highest initial bifidobacterial count of 8.0 lg(CFU/mL). Unlike the bacilli, the metabolism of the test strain affected the synbiotic factor and reduced its value. Probably, the reduction happened because of extra acid production.

The final *Salmonella* count equation ($F = 2.20 < 4.74$, $P = 0.05$) looked as follows:

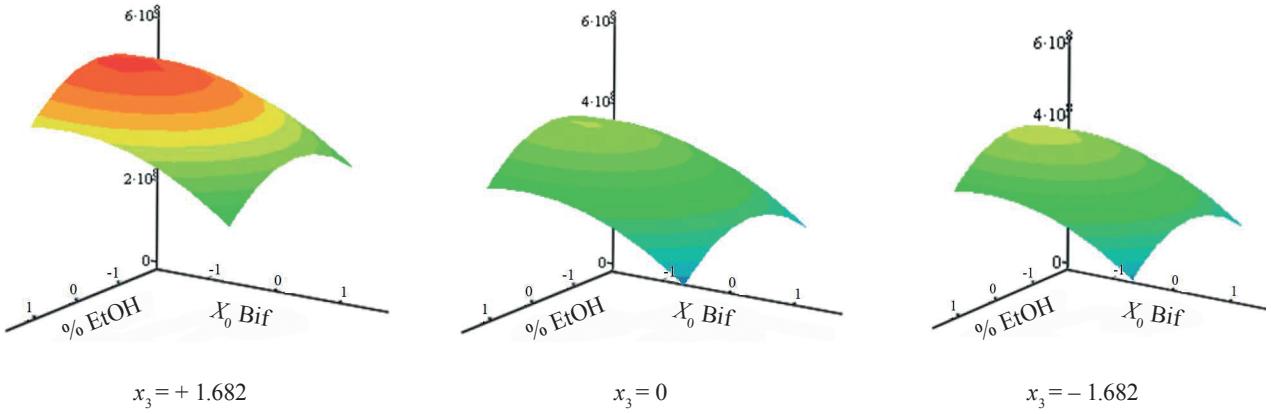
$$Y_2 = 4.14\times10^8 - 4.58\times10^7 x_2 + 3.95\times10^7 x_3 - $$
$$- 3.61\times10^7 x_1^2 - 2.08\times10^7 x_2^2 + 2.63\times10^7 x_3^2 \quad (6)$$

As for the synbiotic factor, all factors had a significant impact on target function Y_2. The response surfaces were calculated for fixed x_3 values (Fig. 4). The surface was parabolic in coordinates x_1 and x_2. The maximal value of the final test strain count was 8.68 lg(CFU/mL) in coordinates 0, –1.101, and +1.685. Thus, the synbiotic composition of fructans precipitated by 50% ethanol and bifidobacteria with the initial count of 6.34 lg(CFU/mL) had the lowest antagonistic effect against *Salmonella*. As the initial *Salmonella* count increased, the efficiency weakened.

The effect of the initial test strain count on the response function was not symmetrical to the design center because the minimum of the function for this variable was at the point – 0.749, 5.83 lg(CFU/mL). The dependence had a quadratic nature. As a result, the final *Salmonella* count remained almost the same when the initial count was below 6.5 lg(CFU/mL). When the values were large, the value of the response function rose sharply. Therefore, the initial *Salmonella* count of 6.5 lg(CFU/mL) was critical from the standpoint of microbiology.

The response paraboloid was symmetrical to the design center of variable. Thus, both fructan fractions precipitated by the highest and the lowest alcohol concentrations possessed the same inhibition effects. However, as the initial probiotic count exceeded 6.34 lg(CFU/mL), the inhibition of the pathogen increased. The lowest values of the final *S. enterica* count (and the greatest antagonistic effect) within the variation range were achieved at the maximal initial bifidobacterial count of 8.0 lg(CFU/mL) in the medium with *A. lappa* root fructan fractions precipitated with 20 or 80% ethanol.

Previously, we considered *Staphylococcus aureus* as the test strain and also found out that the effect of *A. lappa* fructans precipitated with 40 and 60% ethanol was weaker than those precipitated with 20 or 80% ethanol [27]. Apparently, the highest average degree of polymerization was effective because the carbohydrate substrate was less available. The lowest degree of polymerization was effective because the bifidobacteria consumed the substrate faster and thus produced more metabolites. This issue, however, requires further research.



$x_3 = +1.682$ $x_3 = 0$ $x_3 = -1.682$

Figure 4 Final *Salmonella* count response surface as a function of ethanol concentration (x_1) and initial probiotic count (x_2) at fixed initial *Salmonella* count (x_3)

In this study, we considered lactic and acetic acids as inhibitors. As proved by Prosekov *et al.*, many bifidobacteria can produce antimicrobial peptides (bacteriocins), and some representatives of *B. bifidum* are among them [41]. However, their synthesis usually becomes active at the stationary phase, and by that time the bifidobacterial count in the co-culture of bacilli and *Salmonella* stop growing. Therefore, the synbiotic factor calculations did not take into account the effect of bacteriocins. Further research is required to study these inhibitors under conditions close to real, e.g., intestinal simulators with a continuous slow medium flow.

The approach proposed in this paper can also be applied to non-plant prebiotics. Lactulose is one of the best prebiotics [42]. It is often combined with other prebiotics, such as fructooligosaccharides, to make up functional foods. Scientists also turn to oligosaccharides of goat's milk, which are a mix of tri- and tetrasaccharides that consist of glucose, fructose, galactose, and their acylated derivatives [43]. Obviously, the qualitative and quantitative composition affects the action of the prebiotic both separately and as part of a synbiotic composition. Our approach can be applied to similar studies *in vitro*.

CONCLUSION

In this research, the highest synbiotic efficiency belonged to the fraction of fructans with a higher degree of polymerization precipitated by the lowest ethanol concentration and the highest bifidobacterial count. The study established a quantitative relationship between the bifidobacteria and the parameters of fructan production and the antagonistic activity of their synbiotic composition. We also determined the effect of the ratio of probiotic and pathogen counts on antagonism. The proposed approach can substantiate the composition of new synbiotics. In the future, we plan to study other compositions of probiotics and prebiotics *in vivo* to find their optimal ratio.

CONTRIBUTION

S. Evdokimova and B. Karetkin developed the research concept. E. Guseva and I. Shakir were responsible for data curation and formal analysis. B. Karetkin acquired the funding. S. Evdokimova and N. Khabibulina performed the experiments. B. Karetkin and E. Guseva developed the methodology. B. Karetkin supervised the project. E. Guseva and M. Zhurikov worked with the Software. I. Shakir validated the obtained data. S. Evdokimova and M. Zhurikov developed the infographics. S. Evdokimova wrote the original draft. B. Karetkin and V. Panfilov edited the manuscript. All the authors discussed the results and contributed to the final manuscript. All the authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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