Research report

"The probiotic potential of the Ekosynbiotic dietary supplement"

Applies to research carried out in accordance with order no. 271 and 272 of 03/09/2021

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Introduction

According to the assumptions formulated by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are live strains of microorganisms that, when administered in appropriate amounts, modulate the bacterial balance of the intestinal flora and have a beneficial effect on the health of the consumer. Probiotic preparations are increasingly used in supplementation. They are available as a single strain or in a mixture of cultures of different strains that have been selected for their beneficial effect. Probiotic cultures are selected mainly from lactic acid bacteria (LAB), including genera such as *Lactobacillus, Lactococcus, Bifidobacterium;* probiotics are also isolated from *Saccharomyces* or non-pathogenic *Escherichia.*

The basic criteria that must be met for a given strain or product intended for use as a dietary supplement to be considered probiotic are the demonstration of microorganism survival in the conditions of the gastrointestinal tract and antimicrobial activity against undesirable microorganisms, including pathogenic ones. Survival in the conditions of the gastrointestinal tract is not a common feature among the groups of microorganisms mentioned above. It is also a feature that can be controlled; i.e. if the strain does not have the innate ability to survive in the conditions prevailing in the stomach, the strain can be protected in order to maintain its survival in the further parts of the gastrointestinal tract by means of, for example, encapsulation.

The microorganisms that mainly infect the digestive tract are enteropathogenic *E. coli, Salmonella, Listeria.* These are microorganisms that enter the human body from the environment (e.g. contact with animals) or with food. Due to the risk of infection and its effects, these microorganisms have been subject to mandatory monitoring of their occurrence in food (EC Regulation 2073/2005 on food safety criteria). Hence, the high antimicrobial activity of strains considered as probiotics against such a set of pathogens is a very desirable feature.

As part of the received research order, the probiotic potential of the Ekosynbiotyk preparation was assessed, a dietary supplement containing live and active probiotic microorganisms in liquid form. The composition of the preparation is: non-chlorinated water (93.00%), sugar cane molasses (4.60%), blueberry juice (0.60%), cherry juice (0.60%), pomegranate juice (0.60%), a complex of probiotic microorganisms SCD EP11 (0.30%), Jerusalem artichoke (0.15%), rock salt from Kÿodawa (0.15%). The complex of probiotic microorganisms is produced as a result of fermentation involving: *Lactobacillus acidophilus* SCD208, *Lactobacillus plantarum* SCD014, *Lactobacillus casei* SCD469, *Lactobacillus bulgaricus* SCD210, *Lactobacillus fermentum* SCDPPW, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* SCD919, *Bfidobacterium bifidum* SCD521, *Bifidobacterium longum* SCD697, *Bifidobacterium animalis* SCD918, *Streptococcus thermophilus* SCD258, *Saccharomyces cerevisiae* SCD058.

Purpose and scope of the research

The aim of the study was to investigate the survival of microorganisms contained in the Ekosynbiotic dietary supplement in the conditions prevailing in the digestive tract. This feature was assessed in *vitro* in a simulated human digestive tract.
The aim of the study was also to investigate the antimicrobial activity of the dietary supplement, to assess the spectrum of this activity in relation to a set of undesirable strains in the gastrointestinal tract and to assess the scale of this impact.

Material and methods of research

Simulated Gastrointestinal Tract Survival Studies

Quality control of products used for testing

Since the experimental results were monitored and interpreted based on the changes in the number of lactic acid bacteria, total microbial counts and yeast counts, the quality control of the dietary supplement and food products used to simulate the ingesta
included the culture of these samples in the same directions of research.

Simulating the digestive tract – course

In order to determine the survival of microorganisms contained in the analyzed dietary supplement
An *in vitro* model of the digestive system was used (Table 1) to reproduce the digestive conditions in the human digestive tract.

At each stage of the section, pH was measured before and after adding buffers, the tested mixture was incubated at 37.0 ±1.0°C under continuous mixing (60 rpm) on a rotary shaker to ensure uniform distribution of all components and to simulate the movement of food and intestinal peristalsis. Throughout the experiment, samples were taken from each section of the tract and after the food retention time had elapsed. Quantitative cultures were performed for the number of lactic acid bacteria, the total number of microorganisms, the number of yeasts, and molecular analyses of the qualitative composition of the microbial consortium. After the incubation was completed, the colonies grown on the plates were counted and the survival of the tested microorganisms was assessed. The tests were performed in triplicate; the results were averaged.

Samples of digested contents in the small and large intestine were subjected to prolonged incubation (3 days) to assess the development of microbial populations and their potential to colonize these sections of the gastrointestinal tract. For this purpose, during the simulated digestion, contents from the small and large intestine after the physiological digestion time in these sections (2 hours and 18 hours for the small and large intestine, respectively) were collected and subjected to prolonged incubation. The analysis of colonization capacity did not include assessment of the ability to adhere to the epithelium.

Table 1: Characteristic features of the in vitro simulated gastrointestinal tract studies

Products included in the food content	Ekosynbiotic preparation (series ALEA22123821200, valid until 06/05/2023) Still mineral water (total mineralization (mg/l) 742, main mineral components (mg/l) Ca2+ 130.3; Mg2+ 21.9; Na+ 11; HCO3 - 539.1; SiO2 22.1) UHT cow's milk 2.0% fat (nutritional value from the label in (100g): energy value 212 kJ, energy value 50kcal, fat 2g, including saturated fatty acids 1.2g, carbohydrates 4.8g, including sugars 4.8g, protein 3.3g, calcium 120mg; best before date 12/10/2021) Oatmeal (ingredients: apple puree (45%), mango puree from concentrated puree (41%), banana puree (9%), instant oat flakes (4%), apple juice from concentrated juice (1%), vitamin C; nutritional value from the label in (100g): energy value 341 kJ, energy value 81kcal, fat 0.7g, including saturated fatty acids 0.1g, carbohydrates 16.6g, including sugars 13.6g, protein 1.5g, salt 0g, vitamin C 12mg; best before date 10/02/2022)

	Fruit juice (ingredients: juices from concentrated juices: orange (25%), tangerine (25%), water, acidity regulator - citric acid, sweetener - sucralose, vitamin C, natural orange flavour; nutritional value from the label in (100ml): energy value 89kJ, energy value 21kcal, fat 0g, including saturated fatty acids 0g, carbohydrates 5.0g, including sugars 4.9g, protein 0.2g, salt 0g, vitamin C 8mg; best before date 23/06/2022)
Research variants	A – "fasting" variant, the content of the system is the tested preparation and water, pH at the stage of initial digestion in the stomach is 2.5±0.2 B – "after meal" variant, without food matrix, the content of the system is the tested preparation and water, pH adjustment to 4.0±0.2 C – "after meal" variant, the content of the system is the tested preparation and UHT milk, pH adjustment to 4.0±0.2 D – "after meal" variant, the contents of the system are the tested preparation and oatmeal, pH adjustment to 4.0±0.2 E – "after meal" variant, the contents of the system are the tested preparation and multi-fruit juice, pH adjustment to 4.0±0.2 F – "after meal" variant, the contents of the system are mineral water, milk UHT, oatmeal and multi-fruit juice (1:1:1:1)
Episodes digestive tract	Mouth, stomach, small intestine, large intestine
Conditions incubation	37.0±1.0oC; oxygen conditions (sections: oral cavity, stomach, small intestine beginning of digestion) and anaerobic (sections: small intestine, large intestine)
Culture media	**MRS** (de Man, Rogosa and Sharpe), neutral pH (6.5±0.2) – medium intended for determining the number of lactic acid bacteria. **PCA** (Plate Count Agar) – a medium for determining the total number of microorganisms **YGC** (Chloramphenicol Medium) – medium for fungal count analysis **WP** (Peptone Water)
Enzymes and reagents	**Oral cavity:** simulated saliva solution, alpha-amylase solution (Fisher Scientific, 0.29 g/l), digestion time 1 min, pH depending on the use of dietary supplement in the range of 3.4-6.7 **Stomach:** pepsin solution (ALINESS Betaine HCL Pepsin 650/150 mg) in PBS buffer, digestion time 120 min, pH as described above, depending on the gastrointestinal tract variant **Small intestine:** sodium bicarbonate solution 1M, pancreatin solution (Kreon® 10,000 (Solvay Pharmaceuticals) with activity of 10,000 FIP lipase units, 8,000 FIP amylase units, 600 FIP protease units), bile salts 0.4% in the tract volume, digestion time 120 min, pH 6.0-6.2 **Large intestine:** sodium bicarbonate solution 2M, digestion time 18h, pH 7.6-8.0

Antimicrobial activity testing

The analysis was performed according to the USP51 pharmacopoeial method. The modifications introduced consisted in the range of undesirable strains used:

- *Candida albicans* ATCC 10231
- *Candida krusei* ATCC 14243

• *Escherichia coli* ATCC 25922

• *Escherichia coli* M/473/M/16, a strain isolated as a food contaminant • *Listeria innocua* ATCC 33090

• *Listeria innocua* M/475/M/16, a strain isolated as a food contaminant • *Listeria ivanovii* ATCC 19119

• *Listeria monocytogenes* ATCC 13932 • *Listeria monocytogenes* M/468/M/16 • *Pseudomonas aeruginosa* ATCC 10145 • *Salmonella enterica* serovar Enteritidis ATCC 13076 • *Salmonella enterica* serovar Indiana M/472/M/16 • *Salmonella enterica* serovar Typhimurium ATCC 14028 • *Staphylococcus aureus* ATCC 29213 • *Staphylococcus epidermidis* M/476/M/16 and on determining the time points at which the experiment was monitored (0h, 2h, 8h, 24h, 48h, 72h).

Results

Digestive system survival study

Quality control of products used in the experiment

Table 2.: Results of quality control tests for the Ekosynbiotic preparation.

Research Direction	Research Method	[Number, log10 cfu/ml]
Number of aerobic microorganisms Plate method	PN-EN ISO 4833-1:2013-12	8.3
Number of lactic acid bacteria Plate method	PN-EN ISO 15214:2002	8.4
Yeast count Plate method	PN-EN ISO 21527-1:2009	5.1
pH	PB-M-24	3.33

Table 3.: Results of quality control tests of food products simulating food content.

Research Direction	Method Research	Water mineral	UHT milk	Juice multifruit	Oatmeal
		[Number, log10 cfu/ml]			[Number, log10 cfu/g]
Number of aerobic microorganisms Plate method	PN-EN ISO 4833-1:2013-12	<0.10	<0.10	<0.10	<0.10
Bacteria count lactic acid fermentation Plate method	PN-EN ISO 15214:2002	<0.10	<0.10	<0.10	<0.10
Yeast count Plate method	PN-EN ISO 21527-1:2009	<0.10	<0.10	<0.10	<0.10

The required quality of the dietary supplement was confirmed (high number of lactic acid bacteria and their dominant share in the total number of microorganisms) (Table 2). Based on the lack of microorganisms in the remaining food products, their suitability for use in the experiment monitored and interpreted on the basis of changes in the number of lactic acid bacteria, the total number of microorganisms, the number of yeasts (Table 3).

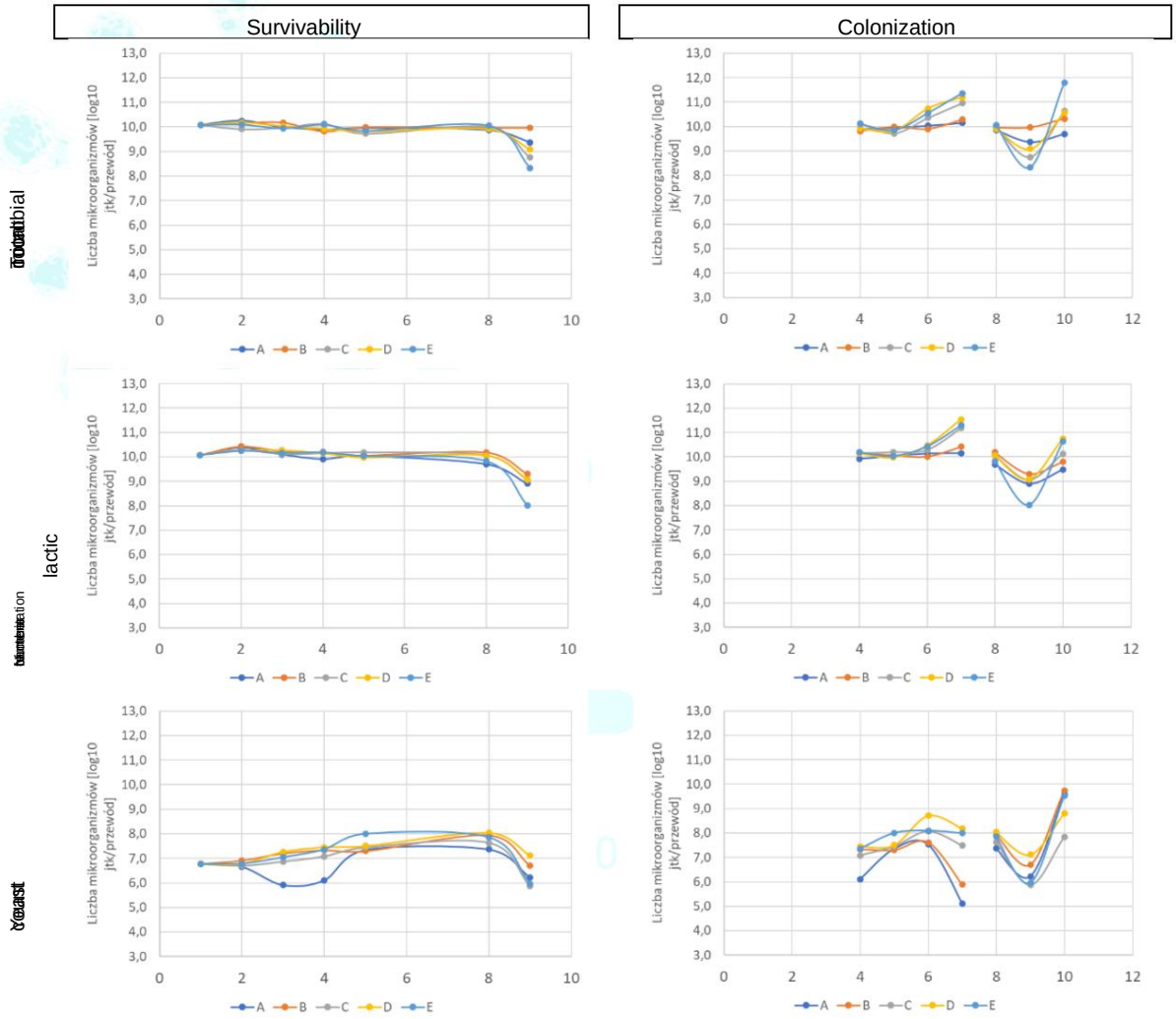
Survival during passage and colonization

The probiotic microorganisms contained in the Ekosynbiotic preparation were subjected to tests to assess:

1. Their survival during the physiological digestive process (taking into account the impact of digestive enzymes, variable pH and environmental composition, including the content of bile salts, taking into account the influence of different food matrix and different conditions of the gastrointestinal tract ("fasting" *vs.* "after a meal");
2. Their potential ability to colonize the gastrointestinal tract in sections particularly important for the interaction between the probiotic and the host organism (small intestine and large intestine).

Table 4.: Survival of microorganisms contained in the Ekosynbiotic preparation in the conditions of the digestive tract in the *in vitro* simulated human digestive tract and changes in the number of microorganisms during prolonged incubation - assessment of the ability to colonize the small and large intestine. Designations: A - digestive tract "fasting", B - digestive tract "after a meal" (only water), C - digestive tract "after a meal" (milk as food), D - digestive tract "after a meal" (porridge with fruit as food), E - digestive tract "after a meal" (fruit juice as food); on the horizontal axes 1 - oral cavity, 2 - stomach, beginning of digestion, 3 -

stomach, after digestion (2h), 4 – small intestine, beginning of digestion, 5 – small intestine, after digestion (2h), 6 – small intestine, prolonged incubation (24h), 7 – small intestine, prolonged incubation (72h), 8 – large intestine, beginning of digestion, 9 – large intestine, after digestion (18h), 10 – large intestine, prolonged incubation (72h).

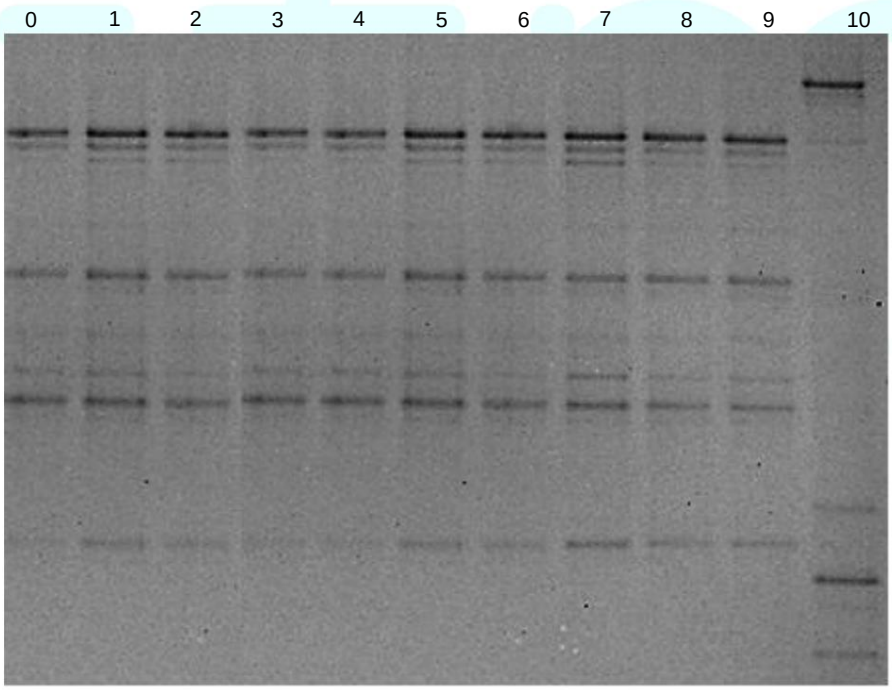


During the survival assessment (Table 4), a moderate decrease in the number of microorganisms contained in the Ekosynbiotic preparation was observed at the stage of digestion in the stomach (applies to lactic acid bacteria) or a significant decrease in the number of yeasts in the same section (applies to the analysis in the digestive tract "on an empty stomach") or an increase in the number of yeasts (applies to the analysis in the digestive tract "after a meal"). After the passage of the food content in each analysis variant (on an empty stomach and after a meal), the number of lactic acid bacteria returned to the initial level, and

the yeast counts were maintained. Only after 18 hours of digestion in the large intestine was a decrease in the counts observed within each microbial group; this should be explained by the depletion of easily accessible food sources in the experimental system.

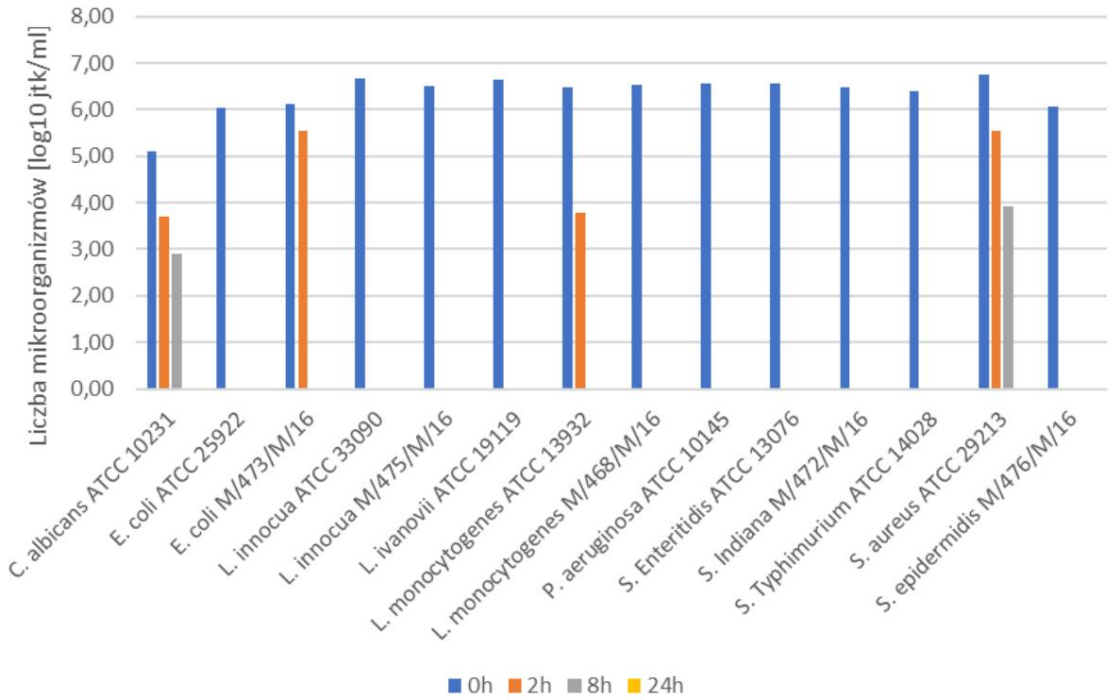
The assessment of the colonization capacity was carried out indirectly based on the assessment of changes in the number of microorganism groups during the prolonged incubation of the experiment in the conditions of the small intestine and large intestine (Table 4). The colonization of the small intestine by the probiotics contained in the Ekosynbiotic preparation is probable; the number of lactic acid bacteria does not decrease (variants "fasting" or "after a meal" with only water) or multiplies (variants with milk, fruit-cereal, fruit food); similar observations concern the number of yeasts.
Colonization of the large intestine is also probable, but it is achieved by selected strains from the consortium (Photo 1), which survived the period of adaptation to the conditions prevailing during the experiment conducted according to the applied methodology.

Photo 1. Molecular study (PCR-DGGE) of the composition of the consortium of microorganisms contained in or derived from the Ekosynbiotic preparation and changes in the composition at subsequent stages of digestion in the gastrointestinal tract under simulated *in vitro conditions.* Designations: 0 – composition of the consortium of microorganisms in the Ekosynbiotic preparation, 1 – oral cavity, 2 – stomach, beginning of digestion, 3 – stomach, after digestion (2h), 4 – small intestine, beginning of digestion, 5 – small intestine, after digestion (2h), 6 – small intestine, prolonged incubation (24h), 7 – small intestine, prolonged incubation (72h), 8 – large intestine, beginning of digestion, 9 – large intestine, after digestion (18h), 10 – large intestine, prolonged incubation (72h).



Antimicrobial activity

Table 5: Antimicrobial activity of the Ekosynbiotic preparation against undesirable strains



Antimicrobial activity was assessed against a wide range of undesirable and pathogenic strains. The results of the study indicate high activity of microorganisms contained in the Ekosynbiotic preparation against all pathogens used (Table 5). Activity against most allows achieving a lethal effect against pathogens already after two hours from the contact of probiotics with the pathogen, which is of great importance for rapid control of infections. In the 8th hour of the experiment, only *S. aureus* ATCC 29213 and *C. albicans* ATCC 10231 are present out of all 15 pathogenic strains. After 24 hours, no live cells of pathogenic microorganisms were detected.

Summary

Na podstawie przeprowadzonych badań i obserwacji zmian liczebności analizowanych mikroorganizmów w symulowanych *in vitro* warunkach, panujących w trakcie trwania w przewodzie pokarmowym człowieka, można wyciągnąć następujące wnioski:

1. Przeżywalność mikroorganizmów probiotycznych zawartych w preparacie Ekosynbiotyk jest wysoka na każdym etapie trwania procesu trawienia.
2. W zastosowanych warunkach trawienia mikroorganizmy probiotyczne zawarte w preparacie Ekosynbiotyk mają wysoki potencjał do kolonizacji jelita cienkiego z jednoczesnym zachowaniem składu konsorcjum minimum do 72 godzin od podania.
3. W zastosowanych warunkach trawienia możliwe jest doprowadzenie do licznej kolonizacji jelita grubego przez zaadaptowane szczepy bakterii fermentacji mlekowej wprowadzone w preparacie Ekosynbiotyk.
4. Preparat Ekosynbiotyk ma wysoką aktywność przeciwdrobnoustrojową wobec mikroorganizmów patogennych. Aktywność ta ma przebieg dynamiczny, doprowadzając do szybkiego wykluczenia obecności analizowanych patogenów.

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