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THE MICROBIAL DIVERSITY AND ITS DYNAMICS IN THE ETHNIC FERMENTED FOODS OF THE BLACK SEA REGION

Homemade fermented food is a valuable source of biologically active prebiotic substances and probiotic microorganisms. Five prioritized ethnic fermented foods from the Black Sea region: Bosa (Bulgaria), Socata (Romania), Kvass (Russia), fermented beans (Turkey) and Sauerkraut (Ukraine), based on the original recipes using unique microbial starters were studied. The dynamics of microbial compositions during the fermentation process of each product was clarified and the best combination of lactobacilli starters needed to reproduce the original formulations were defined. In addition to our earlier published data these results demonstrate the opportunity to design novel food products which might be used in the practical implementation of personalized diets.

K e y w o r d s: ethnic fermented foods, prebiotic and probiotic components, microbial starters, designed food, personalized nutrition.

The fermented products are historically known as a rich resource of pre- and probiotic compounds [1]. Traditional (local) homemade fermented food and drinks were widely investigated for their microbial content in order to propose latest as potential cultures/starters for industrial manufacture [2].

The main problem with their industrial manufacturing is generated by extremely unique conditions of traditional preparation of different local fermented products.

In this work we demonstrate the results of our comprehensive investigation of microbial components' changes during the process of fermentation of traditional for the Black See region countries (BSAC) foods prioritized within the BaSeFood[1] project (http://www.basefood-fp7.eu/).

The main aim of our research was to reveal how these products might be used in a way to design the novel food for the personalized nutrition application – the concept of adapting food for individual needs.

The background to this task lied in the substantial lack of information on the key micro-organisms for food processing of the prioritized fermented foods of BSAC, with special respect to the effects on bioactive compounds' retention and food safety.

Materials and Methods. ***The tasks of microbial analyses*** of ethnical fermented foods that had been performed within BaSeFood project particularly were: (1) to detect and isolate originally used starters (bacterial/yeast origin);

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(2) to define the key microorganisms responsible for the detrimental effect on the quality/safety of fermented foods/drinks; (3) to compare the raw material environmental contamination with the microbial compositions/associations occurring during the main biotechnological stages of the fermentation process; (4) to identify the new species/strains of microorganisms for practical use in the improvement of the safety and/or quality of the chosen composite foods/drinks and their specific influence on the human gut commensal representatives: beneficial species vs. potentially pathogenic isolates of human origin.

Screening the fermented products. In this work we focused on results of microbial assessments of nonalcoholic fermented food and beverages of plant origin (Table 1) during fermentation process in dynamic. Traditional fermented products were selected among of others prioritized foods based on the its definition and documentation [3] as well as consumer's choice revealed via the questionnaire performed during the project execution [4, 5].

Analytical examination. Samples collection and samples handling were described in earlier papers [6–8]. The detailed nutritional composition, minerals, vitamins and bioactive compounds were examined.

Microbial isolation and identification. The major microorganisms were isolated and identified in dynamic from the samples of home-made style vs. industrial-market manufacturing sites fermented foods in order to 1) identify original beneficial starter cultures and 2) investigate the major microbial agents of spoilage/purification processes. Both of these can affect the final quality of the obtained product.

The prioritized fermented products from all the BSAC (Table 1) were tested microbiologically on the 1^{st}, 3^{rd}, 8^{th}, 13^{th}, 19^{th}, 23^{rd} day of fermentation. Temperature for the preparation and storage of Boza, Socata and Kvass was 37 ˚C while for the Sauerkraut and Sautéed pickled green beans it was 20–18 ˚C. All samples of fermented traditional foods were previously homogenized, weighed, titrated in phosphate buffered saline (PBS) for quantitative assay and then plated in 10µl samples at dilutions of 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} on selective and chromogenic media (CHROMagar, France).

Routine microbiological tests were complemented with rapid detection tests (semi- and automatic systems): URI select tests, OXI-tests, API test systems for rapid biochemical identification of isolated microorganisms – API 32E, API NH, API 20 C AUX, API STREP, API STAPH, API 20 NE, API 50 CH, API 50 CHB, API CANDIDA, API CORYNE (BioMérieux, France), and ANAERO test 23 and ENTERO test 24 PLIVA (Lachema Diagnostika s.r.o, Czech Republic); serological identifications were performed additionally using PAST Staphy- and Strep- Latex-tests (Bio-Rad, USA). For the isolation and identification of anaerobes all probes were cultivated on ten different nutrient media, including blood culture medium and an enrichment broth for the isolation of anaerobes.

Table 1

List of tested traditional fermented foods selected based on documentation and questionnaire

Group of prioritized traditional food	No	Fermented food		Country
		English name	Original name	
Low or non-alcoholic fermented foods and beverages of plant origin	1	Millet ale	Boza	Bulgaria
	2	Elderberry soft drink	Socata	Romania
	3	Kvass southern	Квас южный	Russian Federation
	4	Sautéed pickled green beans	Fasulye turşusu kavurması	Turkey
	5	Sauerkraut	Капуста білокачанна квашена	Ukraine

Perfringens Agar (OPSP) was used for *Clostridium perfringens* isolation, MRS was a medium for the cultivation and enumeration of *Lactobacillus spp*.

Orange Serum Agar, pH 5.4 to be a suitable medium for growing *Leuconostoc, Lactobacillus* (*Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum*), and yeasts, such as *Aspergillus niger* and *Saccharomyces cerevisiae*.

Bifidobacterium broth analogues to Blaurock medium for isolation of *Bifidobacterium* species was used, and differentiation of *Bifidobacterium* species was made based on their growth characteristics.

For the isolation of enterococcus species the EN-COCCUS test was used. For the detection of the characteristic features of *Enterococcus* family – the pyrrolidonylarylamidase activity a PYRA test, in the form of detection strips, was also used. Dehydrated Culture Media Brilliance™ Candida Agar (formerly Oxoid Chromogenic Candida Agar, OCCA) was used as a selective differential medium for the rapid isolation and identification of *Candida* species. It comprises three major components peptone, chromogenic mix and agar. Brilliance Candida Agar allows the differentiation of *Candida albicans* and *C. tropicalis* from other species of Candida within 48 hours. BBL™ CHROMagar™ Candida is another selective medium used for the isolation and presumptive identification of yeast and filamentous fungi and differentiation of *C. albicans*, *C. tropicalis* and *C. krusei*.

CHROMagar™ Candida is a selective and differential medium, on which the colonies of *C. albicans, C. tropicalis* and *C. krusei* produce different colours, thus allowing the direct detection of these yeast species on the isolation plate. Colonies of *C. albicans* appear light to medium green, *C. tropicalis* colonies appear dark blue to metallic-blue and *C. krusei* colonies appear light mauve to mauve, flat colonies with a whitish border. Other yeasts may appear light to dark mauve (e.g., *C. glabrata* and other species).

All the measurements were done in triplicates. The statistically verified data presented in this paper.

Results. The samples of **Boza** were collected in the region close to Plovdiv, in the towns of Hissar and Bratsigovo. The popular "Boza" is a thick fermented sweet beverage with a sweet-sour taste prepared from roasted flour, which

gives it a brownish colour. As the beverage is fermented, it has a slight (4 % or less) alcohol content. Millet-flour Boza is preferred, but it may also be made from wheat, barley, oat or corn flour. The dynamic microbial analyses resulted in isolation of various associations of microorganisms on different stages of fermentation. These results are shown on Figure 1.

On the first days of the Boza' preparation the dominant species of microorganisms are *L. fermentum* and *C. pelliculosa* in highest titres – up to 10^9 CFU/ml, accompanied with *B. longum* (10^4 CFU/ml). On the third day of

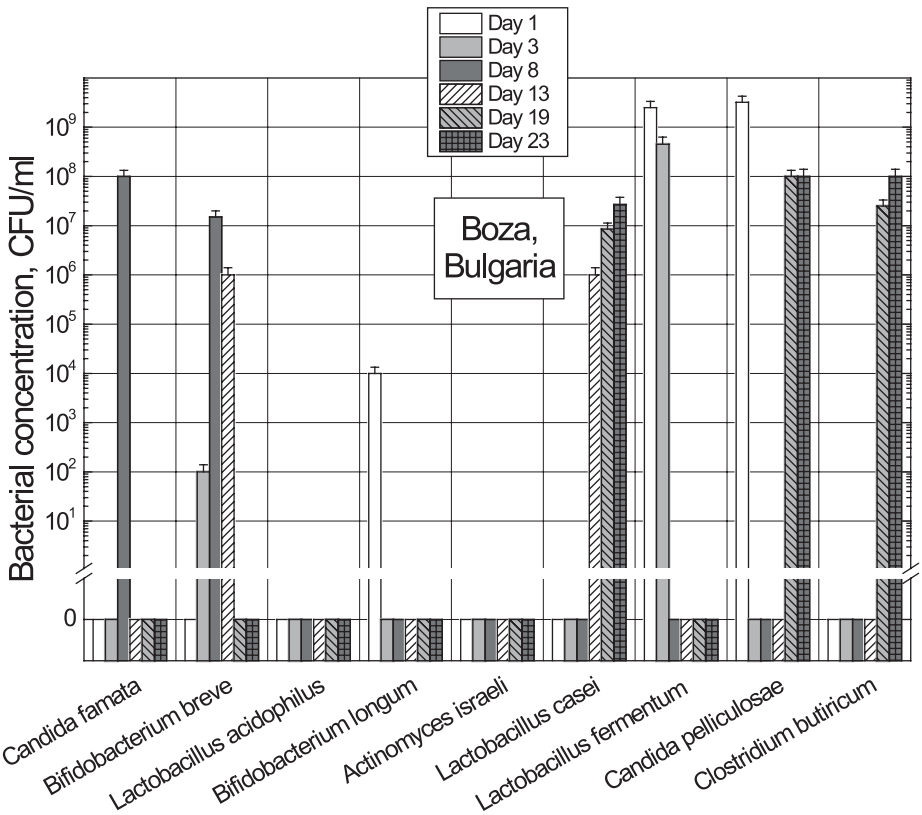

Fig. 1. Microorganisms isolated from Boza during the fermentation process (experiment performed in Plovdiv, Bulgaria)

fermentation these microbial cultures were partially replaced: *B. longum* with *B. brever* (10^2 CFU/ml) and titres of *L. fermentum* was not significantly lower. *C. pelliculosa* was not detected. After one week of fermentation an increased amount of *B. brever* (10^7 CFU/ml) was observed and *C. famata* could be isolated, in concentration of 10^8 CFU/ml. These characteristics are measured for the Boza prepared according its original homemade recipe. It is then usually bottled and stored before being used.

The microbial composition in the bottles was checked on the 13th day of fermentation, and in the two-week old product. The amount of *B. breve* was found not to have significantly decreased (10^6 CFU/ml) and the *L. casei* became the dominant strain among the other lactobacilli detected in almost the same amount as bifidobacteria. On day 19th and 23rd, the three weeks-old Boza product showed an increased amount of *L. casei* (10^6 and 10^7 CFU/ml

correspondingly on day 19th and day 23rd), *C. pelliculosa* in concentration of 10^8 CFU/ml and, for the first time, *C. butiricum* (10^7–10^8 CFU/ml).

The **Socata** (Elderflower Cordial) is a soft fermented drink which is very popular in Romania. It is non-alcoholic in the first 2–3 days and it can become a mild alcoholic drink in 3–5 days (this is the reason why the drink is also known as elder wine). Correspondingly on the third day its content can/should be fixed by filtration through clean muslin, bottled and stored in the fridge. As can be seen in Figure 2, all the isolates had not been found at the beginning of the fermentation process.

Such "biological" sterilization is the result of strong antibacterial and microbiocidic properties of lemon juice and peel added according to the recipe

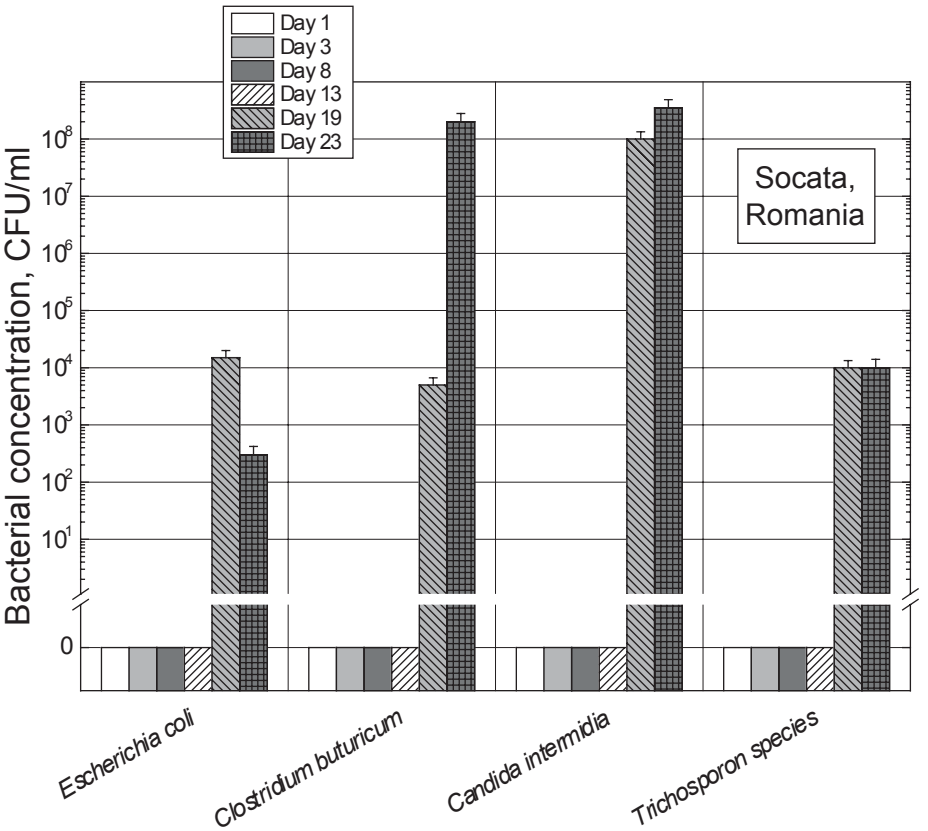

Fig. 2. Microorganisms isolated during the fermentation process from Romanian Socata under experimental condition

and absence of *C. albicans* growth can be caused also by the influence of *S. cerevisae* [9].

Finally, the ready to drink product on the third day is "sterile" even consists of cells of yeast. On the 8th and 13th day, the drink becomes a fermented low-alcoholic product and still had no microbial isolates tested via the applied methodology. Finally, on the 19th and 24th days – two weeks after storage – *E. coli* had been isolated in low concentration – up to 10^4 and 10^3 CFU/ml, *C. buturicum* and *C. intermidia* in titers of 10^8 CFU/ml, as well as strain of *Trichosporon* specie had been also detected in an amount of 10^4 CFU/ml.

Russian Kvass (**Kvass southern**), another fermented drink, was chosen to be studied. Russian Kvass, prepared according to documented recipes [3] from

rye bread, was tested microbiologically in the same way as other fermented products. It had been shown that Kvass is very rich in the variety of isolated beneficial microorganisms – *L. delbrueckii*, *L. plantarum* and *L. casei*, also *B. longum*, *A. israeli*, and *S. cerevisae* TRE-positive strain (Fig. 3).

At the beginning of the fermentation process (1st–3rd days), most of the abovementioned strains were presented in amounts ranging from 10^2

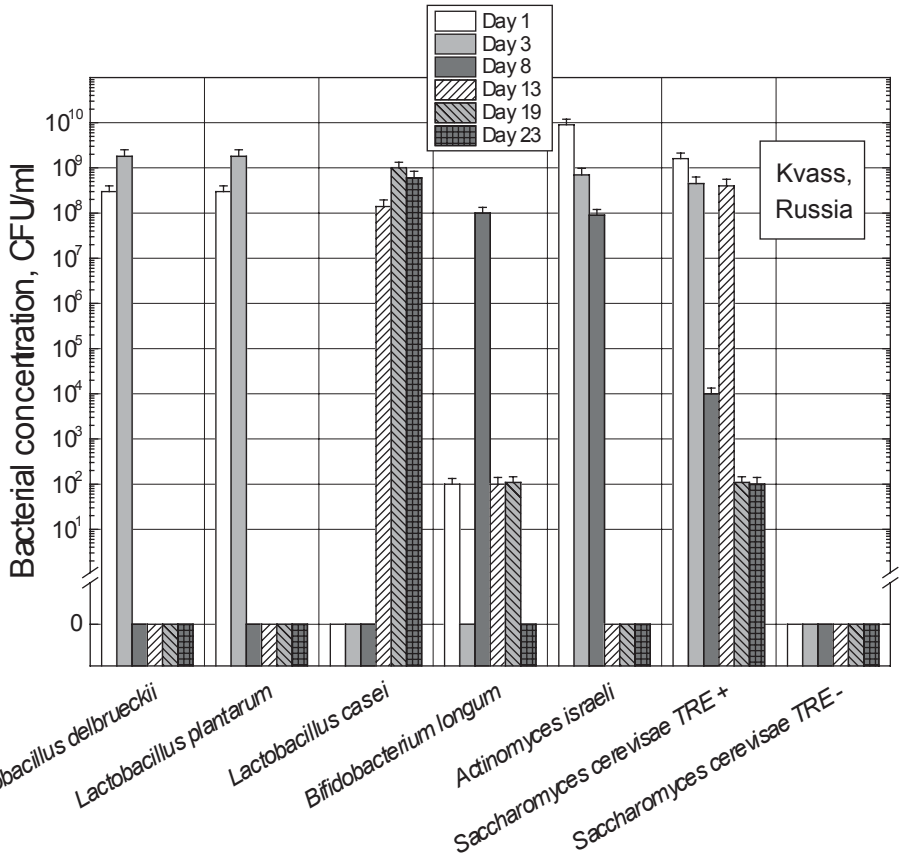

Fig. 3. Microorganisms isolated during the fermentation process from Kvass southern, traditional fermented drink (experiment performed in Moscow, Russia)

CFU/ml for bifidobacteria and 10^8–10^{10} CFU/ml for the other lactobacilli cultures except *L. casei*. After one week, two lactobacilli species – *L. delbrueckii*, *L. plantarum* were not detected, which might have contributed to increasing of the bifidobacteria content up to 10^8 CFU/ml and cause down-regulation of *S. cerevisae* – to 10^4 CFU/ml. These changes are not influenced on titers of *A. israeli*. After two weeks from the start of the fermentation, *L. casei* was isolated in significant titers – 10^8 CFU/ml and were present in Kvass until the end of the experiment. *L. casei* possibly down regulates the content of bifidobacteria to its initial level. TRE-positive *S. cerevisae* TRE-positive and *L. casei* were only detected at the end of fermentation process and *L. casei* dominated being the marker of the spoilage process initiation of the tested fermented product. Bifidobacteria were not present in Kvass at the end of the study (23rd day).

This product differs significantly to other varieties of Kvass investigated microbiologically, both Russian and Ukrainian products obtained in city

supermarkets and industrially produced and bottled compared to the drink obtained from the street-barreled Kvass (Table 2).

As can be seen in Table 2, only a few microorganisms are found in very low amounts in industrial or street produced drink compared to traditional Kvass (Kvass southern). It is unsurprising that these products also differ in taste, colour and other organoleptic properties.

Turkish **Sautéed pickled green beans** and Ukrainian **Sauerkraut** has been also investigated as traditional fermented foods of BSAC within the BaSeFood project.

The first mention above product is one of the many varieties of other local Turkish dishes based on fermentation of vegetables. Pickled cabbage Stir Fry (Tursu Lahana Kavurma) and pickled green tomato Stir Fry (Tursu Domates Kavburmasi) [10] also belong to this category.

The documented recipe as well as a flow chart on how to prepare the Sautéed pickled green bean had been already reported [3].

The difference in a procedure for preparing the fermented beans locally. The beans first were fermented by addition of salt and garlic for several days, like Ukrainian Sauerkraut, *and vinegar was not used*. Dynamic microbiological assay of fermented beans demonstrates the following patterns (Fig. 4).

On the 3rd day, *Lactobacillus spp., C. famata, C. norvegensis (C. lipolytica*) [11], *B. dentinum* and *S. epidermidis* had been found (10^7–10^8 CFU/ml). On the 6th day, significant changes were not observed, only the titers of *C. norvegensis* decreased slightly. The 6th day is actually the last day of the fermentation process.

Table 2

Isolated microorganisms from Kvass received from different sources

No	Kvass	*S. cerevisiae*	*E. coli* lactose +	*L. plantarum*	Origin
		CFU/ml			
1	Kvass	10^2	0	0	Russian market, Moscow
2	Drevlyanskyy kvass (classic) from rye bread	10^3	0	0	Ukrainian market, Kyiv
3	"Yarylo" (wort based on rye, barley malt, corn flour, sugar)	0	10^1	10^1	Ukrainian market, Lviv
4	Lvivs'kyy kvass (with sour-dough of industrial strains of *Saccharomyces cerevisiae* and *Lactobacilli*)	10^1	10^1	0	Kvass street barreled, Uzh-horod

On the 9th day (storage of fermented product) we were not able to isolate *S. epidermidis,* which is eliminated from the tested biomass and all others microorganisms (*Lactobacillus spp., C. famata, C. norvegensis,* and *B. dentinum)* were present in very small amounts – up 10^4–10^5 CFU/ml. The final examination was performed on day 12th of the storage under recommended conditions when some additional microorganisms were isolated – including *C. krusei* and *L. delbrueckii* (both in amount up to 10^5 CFU/ml).

We have tested two samples of ready to eat fermented food **Sauerkraut**, obtained from two different sources: city market and in village where the

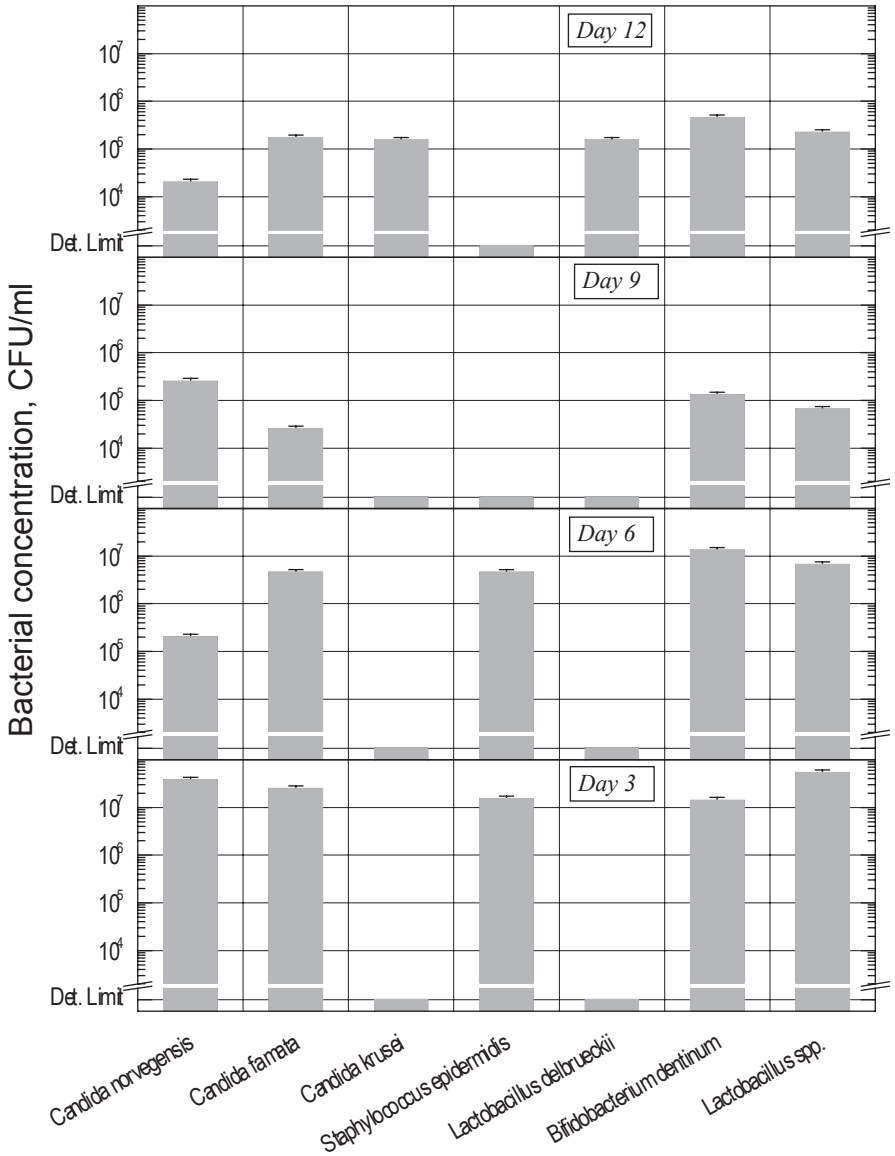

Fig. 4. Microorganisms isolated during the fermentation process from Sautéed pickled green beans (experiment performed in Istanbul, Turkey)

product had been prepared according to traditional technology.

On Figure 5, the differences of quantitative assay for the majority of the most important strains of isolated microorganisms are shown. In homemade samples obtained both from city and villages, the amounts of enterococci strains are minimal and at the limit of detection. In these samples alternatively the increased amount of *Staphylococcus epidermidis* which is known as commensal nonpathogenic bacteria of human skin was detected.

There are no significant differences in titers of bifidobacteria but what is really interesting is that lactobacilli strains belonged to the different species in the tested samples.

Discussion. Controlled fermentation allows to produce designed products enriched with confirmed analytically biologically active compounds and with

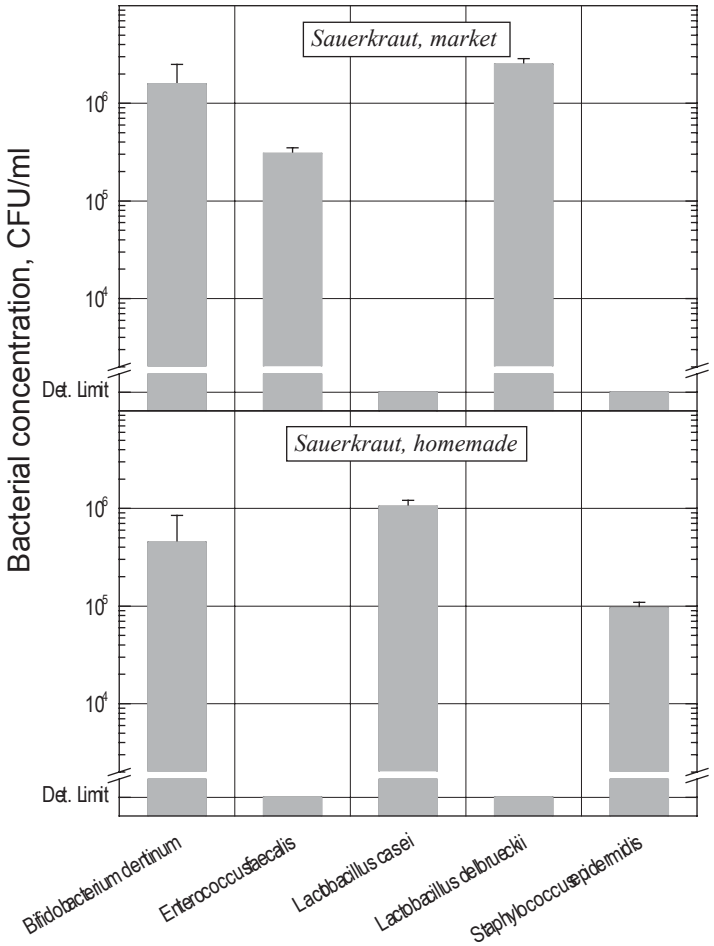

Fig. 5. Microorganisms isolated from homemade Sauerkraut and fermented product obtained in city market

correct combination of beneficial bacteria effecting predictively on human host immune response via modulation of gut microbiota and its metabolites production.

The promising attempts were made to test the effectiveness of designed Boza action against *Mycobacterium tuberculosis* as well as to investigate the properties of its "probiotic" bacteria on different cells models [12].

The only confirmation of beneficial influence of traditional fermented foods and drinks on human health will be accepted in case it can be confirmed by clinical trials randomised double blinded placebo study and up to now only few attempts were made [13, 14]. In the first of them mentioned here, there was no clear evidence detected supporting a clearly positive effect of fermented milk containing three probiotic bacteria on gut symptoms in patients with immune bowel diseases (IBD) compared with the control treatment.

Interventional Randomized Double-Blind Efficacy Study of one low-fat dairy fermented product (drinkable) enriched with plant sterols-esters is supported by DANONE Company and no publications are provided [14].

Microorganisms originated from homemade fermented foods are subject of great importance since they can be potentially used in industrial food processing and as probiotic strains. The LAB strains were isolated from all

investigated fermented foods. Microbial strains possessing strong beneficial properties had been sequenced.

The antibacterial properties have been detected *in vitro* and *in vivo* experiments in lactobacilli strains, isolated from Russian Kvass and Ukrainian Sauerkraut [15], while the strains obtained from Bulgarian Boza and Turkish Sautéed pickled green beans were able to modulate host immune response systemically and locally [16].

Conclusion. There is significant difference between microbial isolates at the beginning, during the process and at the end of the fermentation. We also detected the sufficient changes of variety of microbial species isolated from industrial vs. homemade fermented products. Well-controlled and known processes of preparation of naturally fermented traditional or ethnical foods (TF or EF) is useful for the creation of innovative food (IF) with defined and clinically proved effect on human health being then considered as new generation of functional foods (FF) or designed foods (DF), including personalized nutrition [17, 18].

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РІЗНОМАНІТНІСТЬ І ДИНАМІКА ЗМІН У СКЛАДІ МІКРООРГАНІЗМІВ В ЕТНІЧНИХ ФЕРМЕНТОВАНИХ ПРОДУКТАХ ХАРЧУВАННЯ НАСЕЛЕННЯ КРАЇН ЧОРНОМОРСЬКОГО РЕГІОНУ

Резюме

Ферментована їжа домашнього виробництва є цінним джерелом біологічно активних пребіотичних речовин та пробіотичних мікроорганізмів. Досліджено п’ять пріоритетних етнічних ферментованих продуктів харчування з країн Чорноморського регіону: боза (Болгарія), соката (Румунія), квас (Росія), квашені боби (Туреччина) і квашена капуста (Україна), які відрізняються оригінальністю рецептури приготування та унікальністю мікробних стартерів.

З’ясовано динаміку змін бактеріальних композицій в процесі ферментації кожного продукту, на основі чого відібрано асоціації мікроорганізмів, необхідні для відтворення оригінальних рецептур. Одержані результати досліджень і вже опубліковані нами раніше дані відкривають можливість конструювання нових продуктів харчування, які можуть бути використані при впровадженні персоніфікованих дієт.

Ключові слова: етнічні ферментовані продукти харчування, пребіотичні і пробіотичні компоненти, мікробні стартери, конструювання їжі, персоніфіковане харчування.

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РАЗНООБРАЗИЕ И ДИНАМИКА ИЗМЕНЕНИЙ В СОСТАВЕ МИКРООРГАНИЗМОВ В ЭТНИЧЕСКИХ ФЕРМЕНТИРОВАННЫХ ПРОДУКТАХ ПИТАНИЯ НАСЕЛЕНИЯ СТРАН ЧЕРНОМОРСКОГО РЕГИОНА

Резюме

Ферментированная еда домашнего производства является ценным источником биологически активных пребиотических веществ и пробиотических микроорганизмов. Исследовано пять приоритетных этнических ферментированных продуктов питания населения из стран Черноморского региона: боза (Болгария), соката (Румыния), квас (Россия), ферментированные бобы (Турция) и квашеная капуста (Украина), домашнее производство которых отличается оригинальностью рецептуры и уникальностью микробных стартеров.

Выяснена динамика изменений композиций микроорганизмов в процессе ферментации каждого продукта, на основе чего определены и отобраны комбинации микроорганизмов, необходимые для воспроизведения оригинальных рецептур. Ранее опубликованные нами данные вместе с полученными результатами открывают возможность конструирования новых продуктов питания, необходимых для внедрения персонифицированных диет.

Ключевые слова: этнические ферментированные продукты питания, пребиотические и пробиотические компоненты, микробные стартеры, конструирование пищи, персонифицированное питание.

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