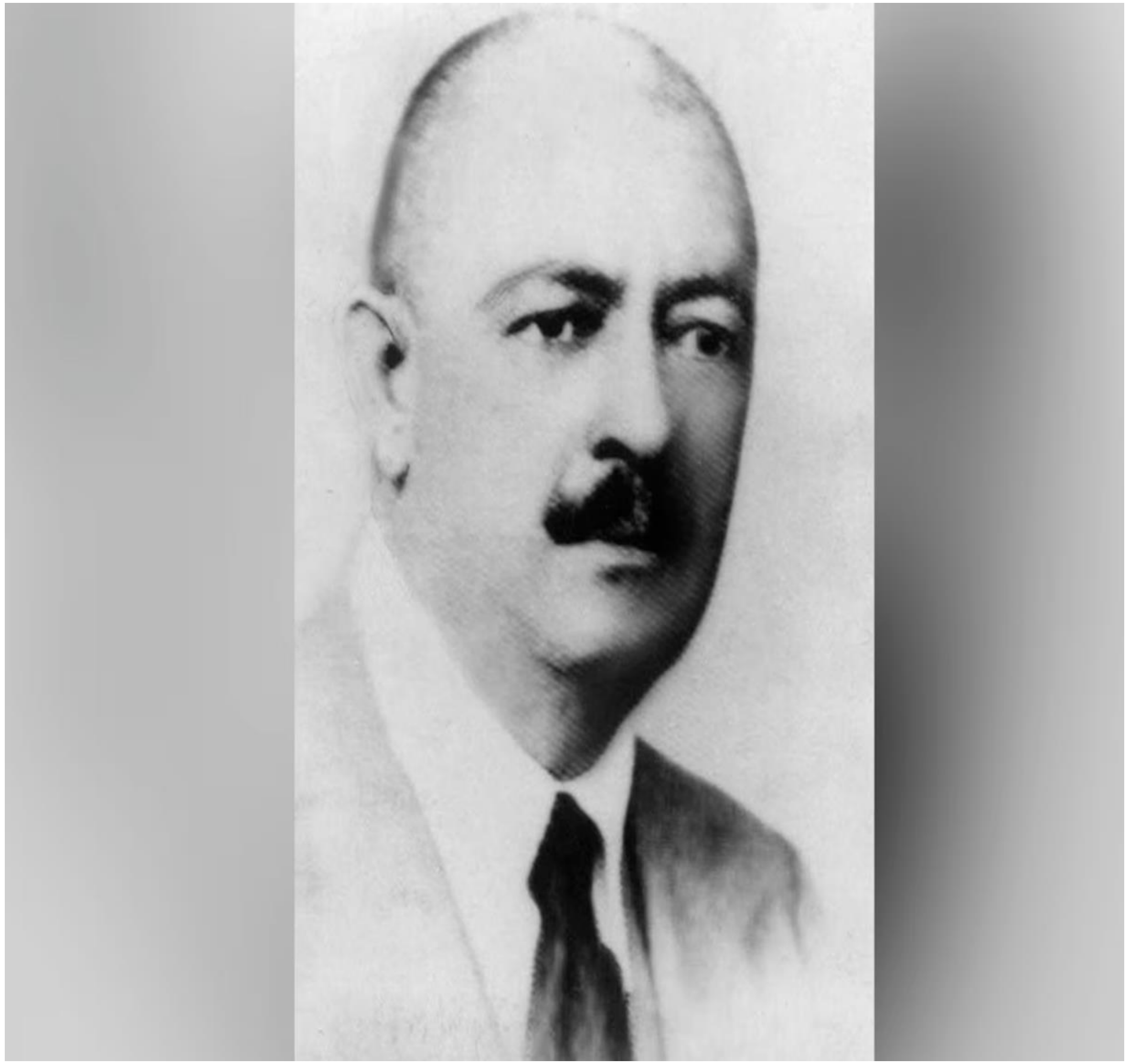
# FUNCTIONAL FEATURES AND CRYOTOLERANCE OF *Lactobacillus* ssp. STRAINS

ТРАКИЙСКИ УНИВЕРСИТЕТ
СТАРА ЗАГОРА

Head Assist. Prof. Dr. Eng. Ivo Ganchev
Trakia University of Stara Zagora
Faculty of Engineering and Technologies of Yambol
Department of Food Technologies
8600 Yambol, 36 Graf Ignatiev street
Republic of Bulgaria
ivo.ganchev@trakia-uni.bg

PASTEUR NETWORK



**Prof. Stamen Gigov Grigorov, 1878-1845**

Production of the traditional yoghurt, white-brined cheese, and yellow cheese, named “kashkaval”, in the Bulgarian region determines everyday consumption and health benefits for the local population.

Dairy products have a special place in Bulgarian diet. After Stamen Grigorov’s discovery, extensive research has begun on the unique nutritional characteristics of Bulgarian yoghurt.

In 1909, the Russian biologist and Nobel Prize winner Elie Metchnikoff developed a theory regarding the prolongation of life. He proposed that there is a relationship between the increased life expectancy of Bulgarians and the daily consumption of yoghurt. Then, it was suggested that the consumption of yoghurt is connected with the increased number of Bulgarian centenarians.

Metchnikoff’s main research was on lactic acid, which is proven to reduce the number of putrefactive microorganisms. Then, he further proposed another hypothesis that the inhibition of pathogens and the harmful fermentation of food in the gut can slow down the process of aging.

Academician Stephan Angeloff (1878 - 1964)

БЪЛГАРСКА
АКАДЕМИЯ
на НАУКИТЕ
1869

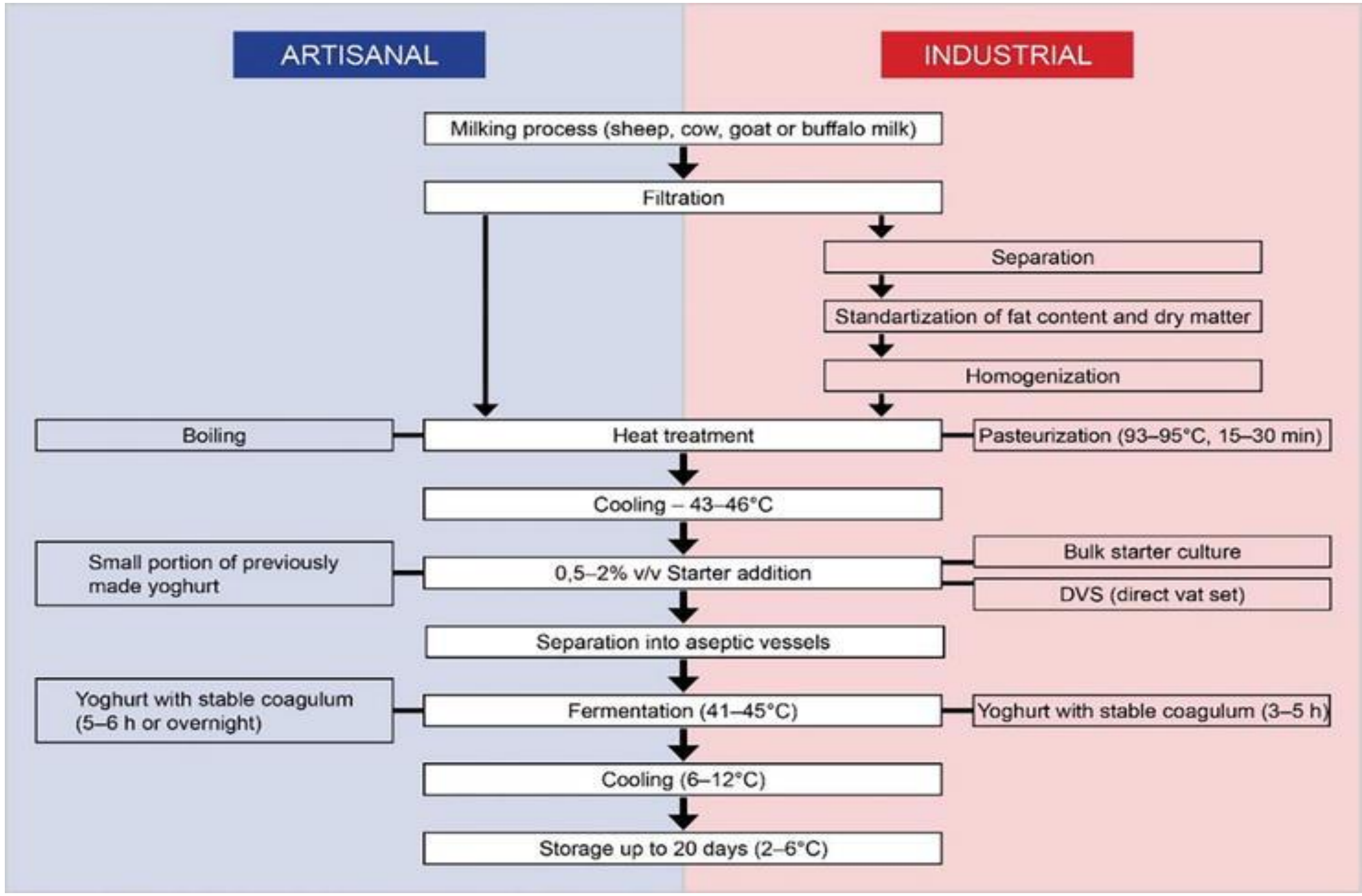
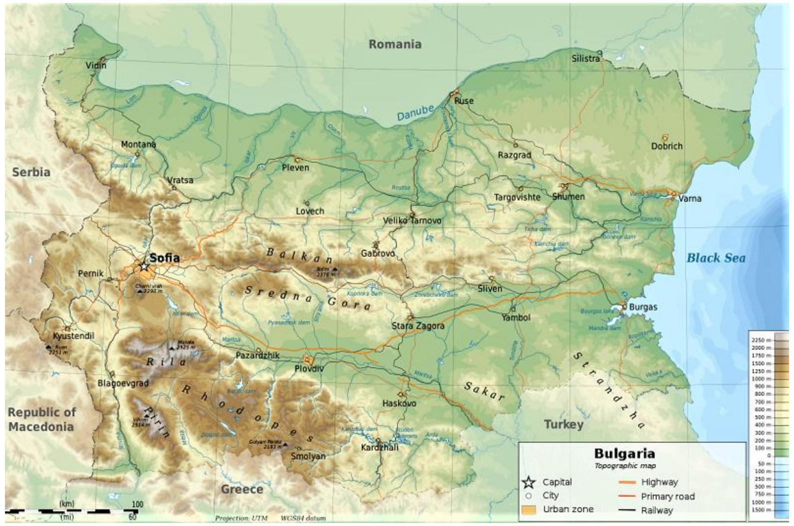



**Figure 1.** Scheme of artisanal and industrial technology of Bulgarian yoghurt production

One of the rarest and almost forgotten Bulgarian dairy products is called *brano mliako*. “It is made in the region of the Rhodope Mountains by an ancient recipe that has remained unchanged for centuries. It is known to be one of the most unique organic foods in the world because of its qualities. “*Brano mliako*” is made from ewe milk and very much resembles yoghurt. The specificity is that it is made only at the end of summer. The technology starts by collecting raw milk in wooden containers, where it is filtered and thickened. The dehydrated milk then ferments spontaneously, or a starter culture can be added to it, mainly sheep yoghurt. After that, a thin layer of goat or sheep tallow is poured on top, so that the product can be “sealed”. This anaerobic preservation makes “*brano mliako*” “suitable to consume for 3 to 4 months.




Gruev (1970) developed a laboratory technology and obtained the same final dairy product. The raw milk is twofold concentrated at reduced pressure at 45–50°C, sterilized by the Koch method for 30 min, and cooled to 45°C. Then, the milk is inoculated with 1% yoghurt starter culture and left for the fermentation process until achieving approximately 190°T acidity. The addition of a 2% yeast-based starter culture, which has been isolated from “*brano mliako*” and cultured in grape must, continues the fermentation. At the end of the yeast fermentation, the obtained dairy product is put in glass containers, hermetically sealed, and stored at 8–10°C for 4–5 months.

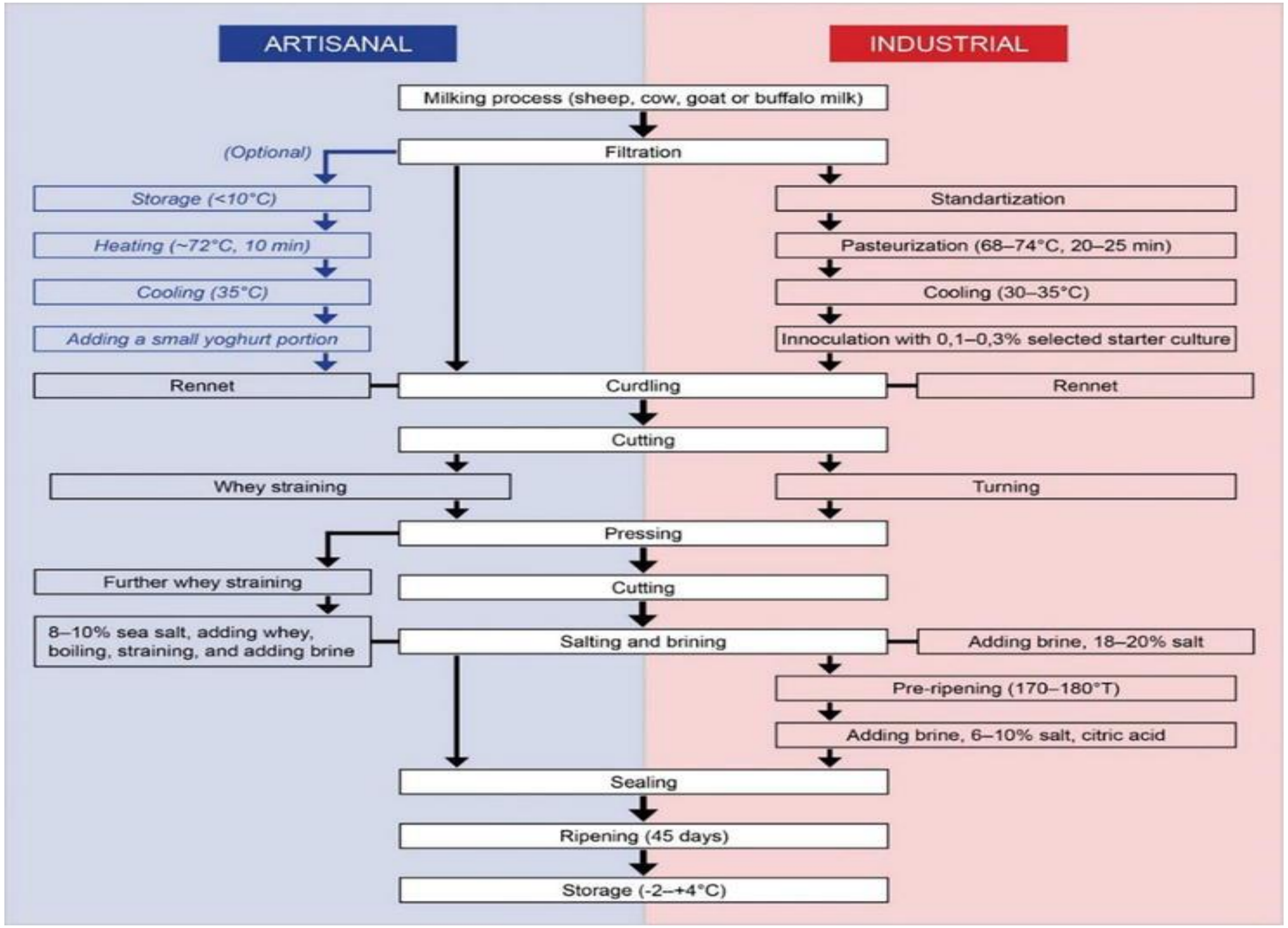
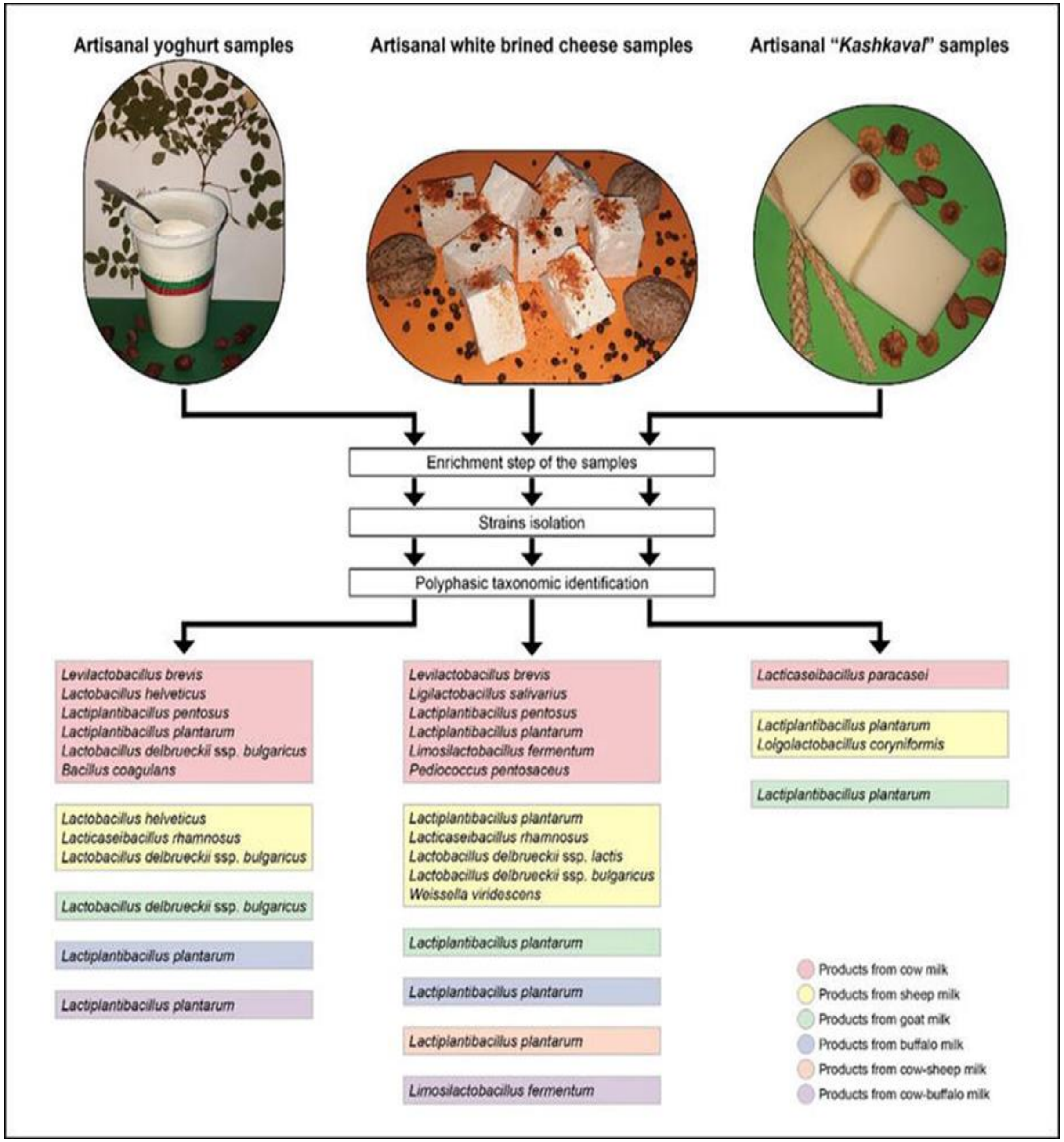



**Figure 2.**Scheme of artisanal and industrial technology of Bulgarian white-brined cheese production.




The diversity of milk, selected starter and nonstarter cultures, and technological treatments give exclusively heterogeneous products with various organoleptic, texture, and nutritional qualities

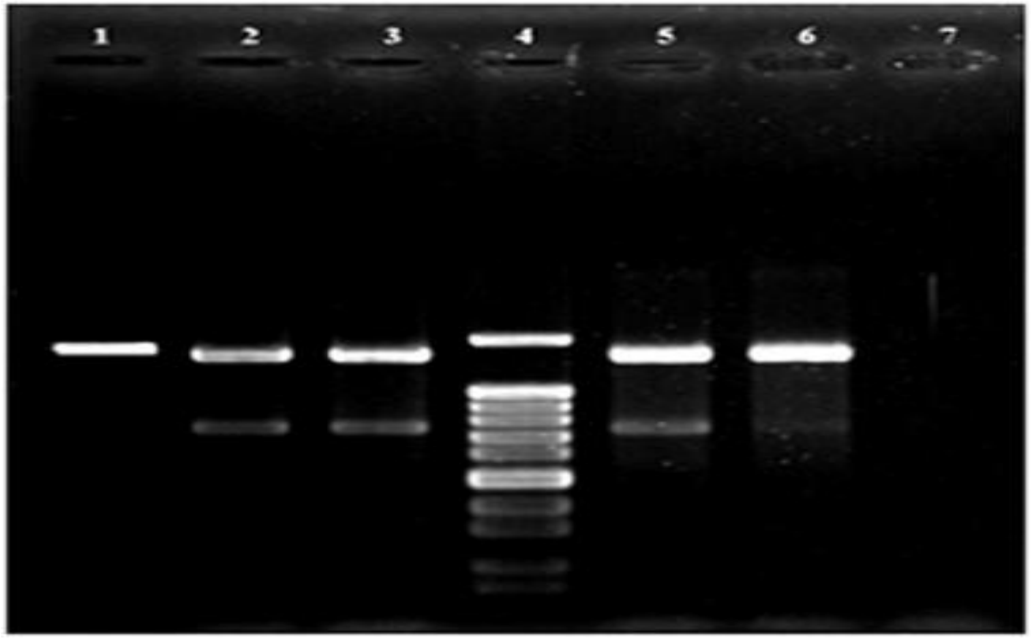
With an understanding of its important role, several studies aimed to characterize LAB diversity, not only in Bulgaria. Discovery of rod- and globular-shaped bacteria (cocci), named initially *Thermobacterium bulgaricum* and *Streptococcus thermophilus*, respectively, started in 1905 with the work of Bulgarian scientist Prof. Dr. Stamen Grigorov.

The cooperation between the two microorganisms was considered to be one of the most important characteristics of the typical Bulgarian yoghurt.

The starter cultures for Bulgarian yoghurt include strains of Bulgarian origin from species *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. LAB reach $10^7$–$10^8$ CFU/g for *L. bulgaricus* and $10^8$–$10^9$ CFU/g for *Str. thermophilus* in the final product.

The biodiversity of autochthonous microbiota and its use as starter cultures make Bulgarian dairy products exceptional. Species from the family *Lactobacillaceae* and genera *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Pediococcus*, and especially the new genera of previously determined genus *Lactobacillus* have been isolated throughout the years from different homemade and artisanal dairy products. The autochthonous microbiota of "*brano mliako*" is comprised of yeast species as well, including *Kl. lactis* and *Saccharomyces cerevisiae*.

The combination of classical phenotypic and microbiological with molecular and typing methods, according to the polyphasic taxonomic approach applied, allowed us to establish the biodiversity of their LAB microbiota. The most persistent was the species *Lactiplantibacillus plantarum.*




16S rDNA sequencing, *L. bulgaricus* was identified in yoghurt samples. In addition, *L. plantarum* presence in yoghurt with a dominance in ripened samples of cheese was confirmed. In the early stage, however, significant number of enterococci and lactococci have to be pointed for white-brined cheese samples. Originally, *lactobacilli* have a low density in cheese (<50 CFU/g). During the ripening period, they significantly increase and become the dominating microbiota in the final product ($10^7$–$10^8$ CFU/g). Therefore, an enrichment step with pre-culturing of collected samples allowed us to establish LAB diversity.

According to Bulgarian National Standard for white-brined cheese, a mesophilic and/or thermophilic starter is used in a ratio of 2:1. The mesophilic starter is predominant and contributes to the ripening processes and developing the taste and aroma of the final product. The mesophilic LAB for white-brined cheese and "*Kashkaval*" are represented by *Lactococcus lactis* subsp. *lactis* and *Lacticaseibacillus casei*. The thermophilic starter includes *L. bulgaricus* and *Str. thermophilus*. For yellow cheese "*Kashkaval*", in addition to *L. bulgaricus* and *S. thermophilus*, the thermophilic *Lactobacillus helveticus* is also present. For all Bulgarian dairy products, the strains in the starters must be isolates of Bulgarian origin.

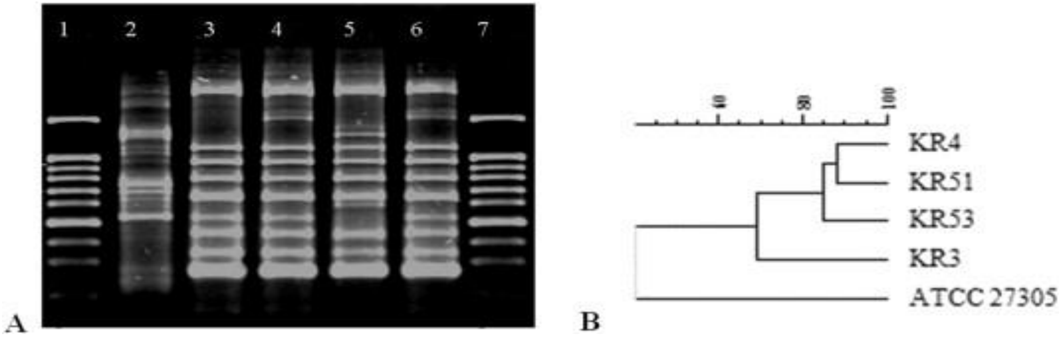



**Figure.** DNA fingerprints obtained with M13V primer in RAPD-PCR analysis and visualized in 2% agarose gel, after ethidium bromide staining: 1 – GenLadder 100 bp + 1.5 kbp (Gennaxon, Germany); 2 – *L. brevis* ATCC 27305$^T$; 3 – KR53; 4 – KR4; 5 – KR3; 6 – KR51. (B) A dendrogram derived from computer cluster analyses of the digitalized images.

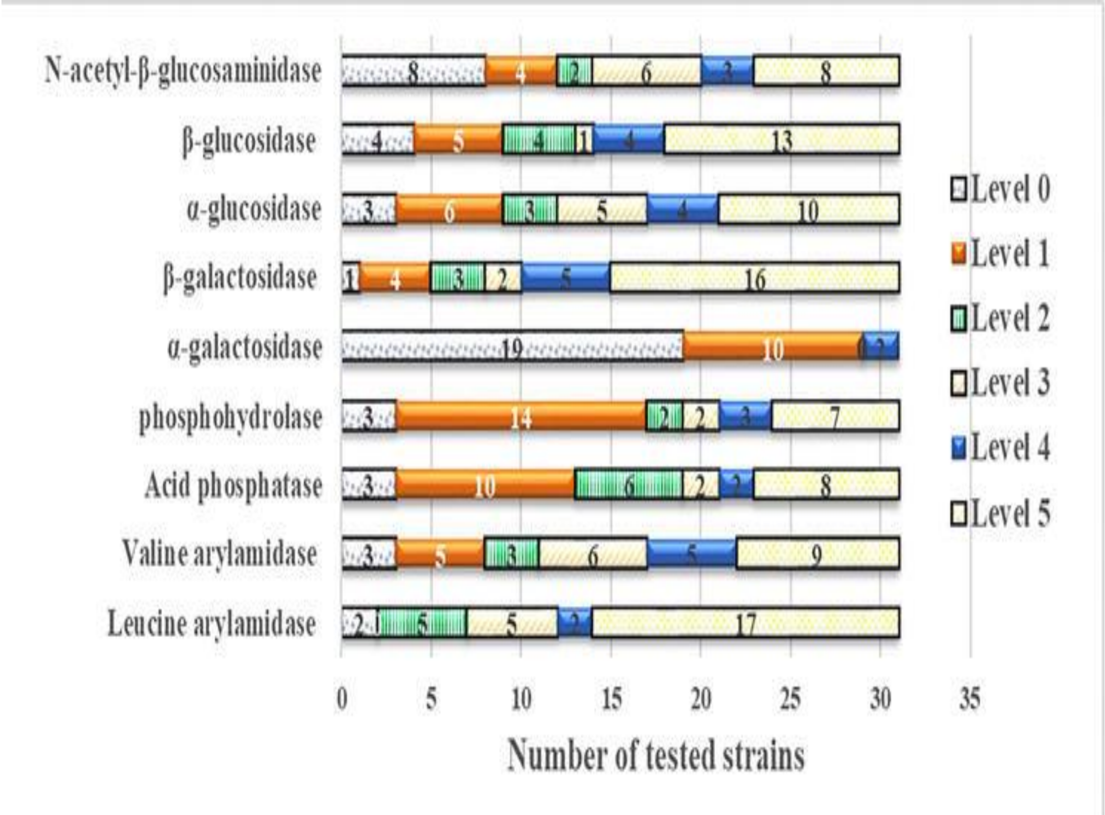
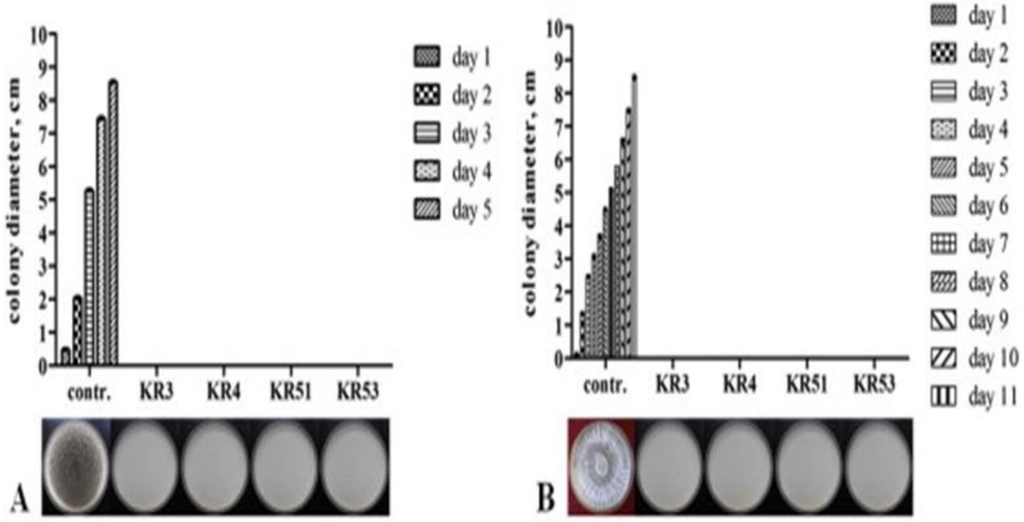



**Figure 4.** Enzymatic activity of 31 *L. plantarum* strains isolated from artisanal Bulgarian dairy products (semi-quantitative analysis with the API ZYM test strips, bioMérieux, France).

The produced LAB enzymes responsible for lipolysis and proteolysis in milk are among the key factors for the sensory qualities of taste and texture of cheese. LAB possess different degrees of lipolysis, which is important for the selection of strains that can be used for starter cultures. During lipolysis, triglycerides hydrolyze into mono- and diglycerides, free fatty acids, and glycerol. The reduced glycerides participate in coagulation processes with different components of dairy foods that lead to the texture development of the final product. This characteristic is related to the flavor development of fermented dairy products

During proteolysis, hydrolysis of the protein peptide bonds occurs and transforms them into peptides and free amino acids. Although many LAB are considered to have weak proteolytic activity, they possess complex proteinase/peptidase systems comprising peptidases on the cell wall initiating the degradation processes, peptide transporters, and intracellular peptides that break down peptides into shorter molecules and free amino acids. Thus, essential amino acids may have accumulated in fermented products, due to the high peptidase activity estimated for *L. plantarum* strains. For 8 of tested L. plantarum strains from white-brined cheese, accumulation of free amino acids from 0.170 to 0.609 mMGly/L was shown. The accumulated free amino acids are involved in reactions of deamination, transamination, decarboxylation, and desulfurization with an impact on the flavor profile of the end product. At the same time, these 8 strains were characterized by low proteolytic activity.

Peptidase as well as β-galactosidase activity is promising for candidate probiotics, while other enzyme activities may contribute to organoleptic characteristics and stability of the products.




Antifungal activity of skimmed milks, fermented with *L. brevis* KR3, KR4, KR51, KR53, against *Aspergillus awamori* (A), *Penicillium claviform* (B), as determined by hyphal radial growth inhibition after 5–11 days of incubation at 29 °C compared with a control. Results are presented as mean (SD), *n* = 3.

LAB can be used not only as starter cultures but also as protective cultures to improve the safety and/or shelf life of the product.

LAB produce a large range of antimicrobial substances, including organic acids (lactic, acetic, etc.), fatty acids, antifungal peptides, reuterin, and bacteriocins.

The metabolites produced by LAB can be divided into two main groups: substances with a low molecular mass < 1000 Da and substances with a high molecular mass > 1000 Da, such as bacteriocins/bacteriocin-like inhibitory substances (BLIS).

As bioprotective cultures, LAB are expected to possess antibacterial/antifungal activity that is exhibited and maintained throughout the manufacturing process and storage time, to have no impact over the functions of the starter cultures, not to modify the organoleptic properties of the final product, to be used with the lowest possible inoculum that maintains the same activity to reduce the cost value, and to have easy propagation and resistance to technological processes. In the later stages of ripening, *lactobacilli* are well adapted to the environment inside the cheese, withstanding the low pH, high salt concentration, absence of sugars, and anaerobic conditions.

Initial results with *L. plantarum* strains from white-brined cheese were highly promising. In the presence of 5% (v/v) CFS from 12 newly isolated strains from cheese and yoghurt cultures, the growth of *Pseudomonas aeruginosa* PA01 (from a patient with cystic fibrosis) was significantly inhibited up to 50–80% (unpublished data). A group of active strains belonging to the species *L. plantarum* and *L. rhamnosus* from dairy products inhibited *Staphylococcus aureus* MRSA as well as out-patient antibiotic-resistant strains.

*L. bulgaricus* possess high antimicrobial properties and are able to colonize the human intestines, which suggests its probiotic functions. More studies describe that regular consumption of yoghurt that contains viable *L. delbrueckii* and *Str. thermophilus* can improve decreased lactose intolerance and the overall digestion of lactose. From intensive research, Bulgarian yoghurt could be considered a dairy product with functional characteristics, as scientific results state that functional probiotic foods can modulate the microbial composition in the gut, thereby improving intestinal health.

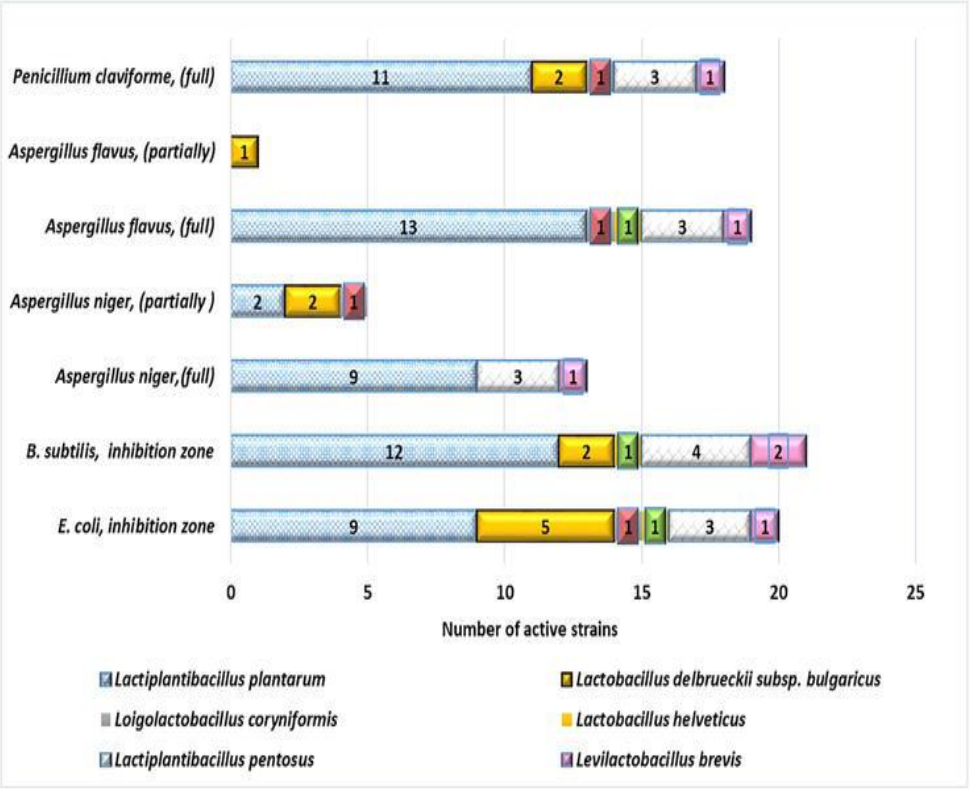



**Figure.** Antagonistic activity of strains isolated from artisanal Bulgarian dairy products against food-associated pathogens and contaminants.

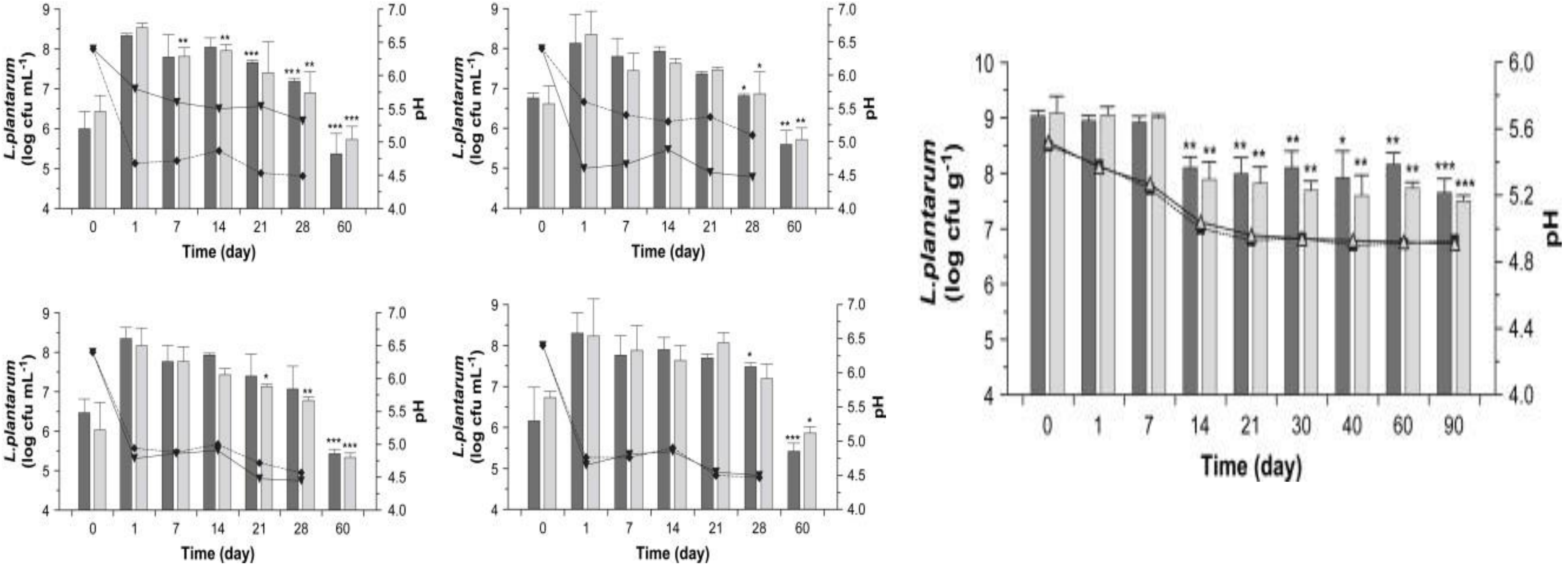



Figure. Viability of *L. plantarum* strains and *L. plantarum* RL34 (, ) and pH change ( ▾ ; ♦) of fermented milk during cold storage. Values are means (±SD) of three replicate evaluations for each bacterial strain; t-test: *p < 0.05; **p < 0.01; ***p < 0.001.

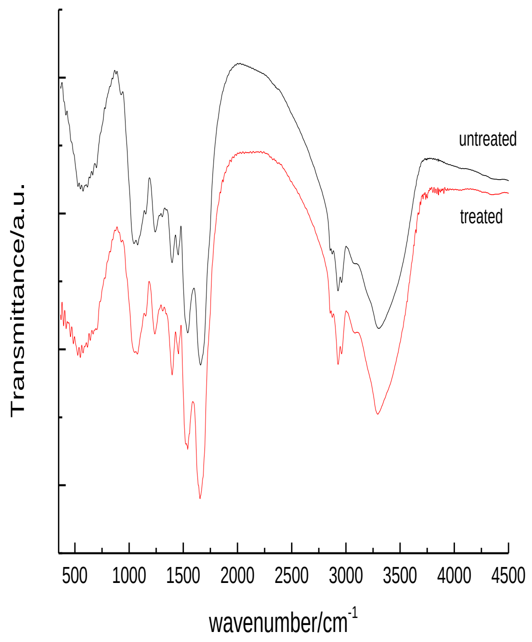



Fig. 1. FTIR spectra of the raw (Sp) and termal (red line) treated *Spirulina platensis* biomass at the wavelegh in the range of 450 nm to 4500 nm (wavenumber on the figure)

Freeze drying has been used for the preservation and storage of probiotic lactic acid bacteria. The selection of an appropriate drying medium is very important in order to increase their rate of survival during drying and subsequent storage

In the present study the cryotolerance of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 was improved by adding of *Spirulina platensis* biomass at the concentration of 12 % in the medium before freeze-drying process.

It is possible that the three mechanisms of direct interaction of the sugar moiety with the polar residues of macromolecules, formation of glasses in the dried state (vitrification hypothesis) and exclusion of sugars from the surface, which may concentrate residual water molecules close to the biomolecular surface, operate simultaneously during the dehydration-rehydration processes.

*Spirulina platensis* biomass may maintain the levels of residual water necessary to preserve the cellular structures from damage in the freeze-dried microorganisms, thus allowing for an appropriate preservation and avoidance of unnecessary damage.

According to our results, *Spirulina platensis* biomass containing the highest proportion of carbohydrates (13.6%), some of which are glucose, rhamnose, mannose, xylose and galactose, were the most efficient in the protection of cells during desiccation at 12% concentration

This minor efficiency of *Spirulina platensis* biomass was due to the high water absorption capacity, due to the higher content of starch and proteins with polar amino acid resides, making them hydrophilic

The slight difference in the protection effect mediated by *Spirulina platensis* biomass at concentration in the range form 10% to 12% might be due to intrachain H-bond networks.

The physicochemical feature of the structures of algae-based biomass also promotes his interaction with external ions and inter-chain H-bonding

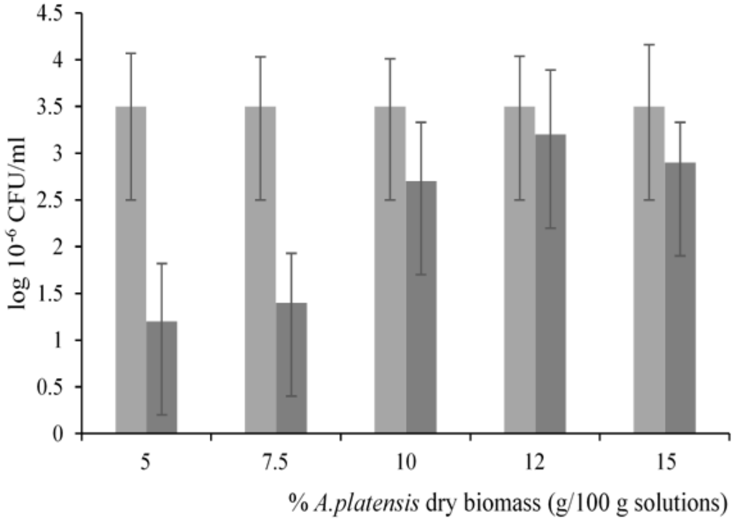


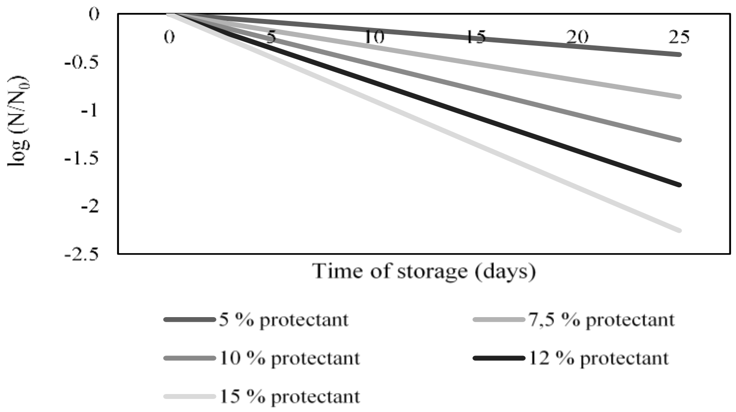

Fig. 2. Colony Forming Units as log10 of *Lactobacillus bulgaricus* WDCM 00102 strain recovered after desiccation over *Spirulina platensis* biomass at different concentrations of *Spirulina platensis* biomass in the cryoprotective media. Counts of microorganisms before desiccation (control) (□) and desiccated in the presence of *Spirulina platensis* biomass (■).

When stored at 4 °C, the survival of *L. bulgaricus* WDCM 00102 freeze-dried in the presence *of Spirulina platensis* biomass at the concentration for 12% was high, i.m. the constant of viability loss over 15 days was changed in the narrow range of 0,0170 ± 0,004 to 0,0190 ± 0.002.

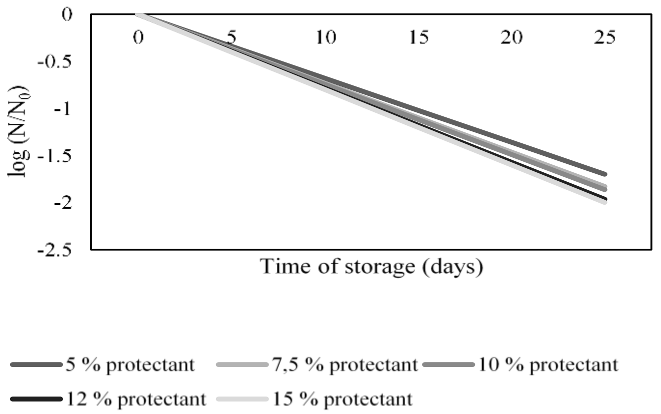
The increasing the storage temperature to 25 °C resulted in a high level of mortality, specifically the constant of viability loss were characterized by the value of 0,0680 ± 0,005 on the 5 day of storage and changed up to value of 0,0800 ± 0,010.

The rate constant (k) of microbial inactivation, expressed as the logarithmic value of viable cells at the beginning of storage ($N_0$) and at a particular storage time (N), was changed linear in the relation to the concentration of *Spirulina platensis* biomass during storage of frezze-dried *Lactobacillus bulgaricus* WDCM 00102 strain at temperature of 5 or 25 °C for 25 days.

The k values also indicate that cryoprotection mediated by *Spirulina platensis* biomass 12 % (storage temperature: 5 °C) was the best condition for storage.




A




B

Fig. 4. Survival of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 after freeze-drying in the presence of *Spirulina platensis* biomass and stored at 5 or 25°C. The $N/N_0$ vs time after freeze-drying was plotted for all the conditions assayed. N: CFU after storage, $N_0$: CFU after freeze-drying and before storage. (A) Freeze-drying in the presence of *Spirulina platensis* biomass at 5°C ($P < 0.05$); (B) Freeze-drying in the presence of *Spirulina platensis* biomass at 25°C ($P < 0.05$).

# CONCLUSIONS

In regard to their capacity to grow in milk after rehydration *Spirulina platensis* biomass was more efficient at minimal concentration of 12% in the recovery of *L. bulgaricus* WDCM 00102 after damage and for low temperature storage.

In this work, we have demonstrated that *Spirulina platensis* biomass is very efficient in the cryopreservation of *L. bulcaricus* WDCM 00102 at 12 % concentration. Considering the physico-chemical and nutritional properties of *Spirulina platensis* biomass, their interaction with probiotics may be useful for the development of commercial synbiotic products, which could be incorporated into different foods (i.e. infant formulas, powders containing probiotics in combination with prebiotics), which may be useful as functional food ingredients for the manufacture of probiotic foods.

# TRAKIA UNIVERSITY OF STARA ZAGORA, REPUBLIK OF BULGARIA

Trakia University is a centre for education, science and spirituality, a place for building specialists and scientists in all fields of knowledge.
It occupies a prestigious place in the top ten of the ranking of universities in Bulgaria and is an example of an international and multidisciplinary institution.

Its structure includes the following units: Faculty of Agriculture, Faculty of Veterinary Medicine with University Veterinary Hospital, Faculty of Medicine with University Hospital, Faculty of Pedagogy, Faculty of Economics, Faculty of Engineering and Technology - Yambol, Medical College - Stara Zagora, Branch - Haskovo, Department of Information and Teacher Training, as well as Academic Technology Complex, Institute for Sustainable Transition and Development, Institute of Food Security and Institute of Zoonoses "Open Health".

# Faculty of Engineering and Technologies of Yambol

Faculty of Engineering and Technology – Yambol is the successor of important educational institutions for the city, training personnel with higher and secondary education in the field of technical and pedagogical sciences. The educational institution was established on September 1, 1964, as a branch of the Institute for Specialist Teachers “Kalinin” in the city of Sliven. The training is in one specialty – “Agricultural Mechanization”, in the building of the Technical School for Agricultural Mechanization. Its foundation is connected with the urgent need at that time for pedagogical personnel for agricultural schools, which forced the state and municipal authorities to open a higher educational institute on the territory of Yambol district, which would prepare teachers specialists in agricultural machinery and vehicles.



By Decree No. 308 / 17.11.2011, State Gazette No. 93 / 25.11.2011, Technical College - Yambol was transformed into the Faculty of Engineering and Technology within the structure of Thracian University - Stara Zagora.

The Department of Food Technologies was established as an independent department by a decision of the Academic Council of Thrace University dated 19.12.2012.



The department trains students in:

specialty "Food Technology" for the Bachelor's degree;

specialty "Food Safety and Quality" for the Master's degree.


Two doctoral programs are also accredited to the Department of Food Technology:

"Technology of Animal and Vegetable Fats, Soaps, Essential Oils and Perfumery and Cosmetics";

"Food Technology".

Main scientific directions in which the department works:

- Use of the natural potential of herbs and spices in the food, pharmaceutical and cosmetic industries;

- Innovative technologies for the production of functional, healthy and dietary foods;

- Ecologically clean and safe food resources and foods;

- Technologies for obtaining bioproducts: enzymes, antibiotics, protein products, vitamins, etc., and their application in the food industry and agriculture.



VELICE DĚKUJI!


THANK YOU
VERY MUCH!