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FERBEV

Improving the Processing of Four Fermented Beverages from Eastern European Countries

Co-operative Research Project

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TABLE OF CONTENT

1 Project execution

1.1 Summary overview

The project aims to improve three traditional fermented beverages and implement the production of an innovative soy-based fermented drink.
These products are *Boza*, a Turkish cereal-based beverage, *Ayran*, a Turkish mildly-fermented milk-based, consumed as a drink after water and salt addition, and *Matsony*, a Georgian milk-based beverage; these drinks usually contain a high number of viable microorganisms. Additionally, a *Fermented Soy Beverage* developed in Poland has been the object of analyses, standardisation and implementation into production in one SME established in Czeck Republic.
The Strategic Objectives of the project, regarding the improvement and standardization of these fermented beverages, are: 1) to acquire a comprehensive understanding of these drinks, 2) to modernize the existing fixtures, 3) to standardize the production of beverages and extend their shelf-life, and 4) to acquire information about the potential beneficial effects on human health of the microorganisms and chemical components present in these drinks.
The project involves **seven SMEs** (beverage manufacturers, technologies and plant suppliers) and **six RTD** public bodies and institutes, who have studied and characterized fermented beverages and from their natural microflora have collected about **eight hundred microbial colonies** from different steps of each drink production; colonies have been isolated, identified, and biotyped by molecular and biotechnological techniques. A high microbial diversity at species and strain level was found in *Boza* whereas a lower biodiversity was detected in *Ayran* and *Matsony*. The new *Fermented Soy Beverage* was produced by using starters including two lactic acid bacteria and one *Bifidobacterium* strain.
Interestingly, bacterial strains endowed with acidifying, proteolytic activities and/or ability of exopolysaccharide production as well as antimicrobial activity against pathogens were selected, and yeasts able to turn L-phenylalanine into a rose-like flavour were highlighted. A **FERBEV microbial culture collection**, including more than 200 strains of bacteria and yeasts, was set up.
Boza heat and drying treatments lowered microbial cell counts; in this way **shelf-life** of ***Boza*** was extended up **to six months**, as planned. Good sensory characteristics and high probiotic viable cell counts were found in *Fermented Soy Beverage* stored at 4°C for 30 days. Shelf-life of ***Ayran*** and ***Matsony*** was extended up to **one month** under correct production and storage practices.
More than twenty autochthonous microbial combinations were tested as starters for *Boza, Ayran, Matsony and Fermented Soy Beverage* under laboratory conditions. On the basis of the results, **two selected starters for each beverage** were chosen and successfully used to manufacture drinks with the **pilot-plant appositely set up** in the FERBEV project.
Chemical, microbiological and sensory analyses of the beverages were carried out for at least six weeks on the cold stored beverage samples. As concerns ***Boza***, the two autochthonous selected **starters** had a **positive impact on shelf-life** that was **longer than 42 days** under cold storage. In addition, *Boza* revealed a low and stable ethanol content and viscosity during all storage. As concerns ***Ayran,*** microbiological and chemical features were **stable up to 42 days** and sensory analyses showed a good consumer acceptance up to 30 days of beverage cold storage. Very interesting results were obtained as concerns ***Matsony*** in the course of the project the cooperation of beverage producer, with the other partners allowed to modernize its equipment, and improve the hygienic manufacturing conditions. Furthermore, the use of selected autochthonous starters allowed to produce samples that maintained chemical, microbiological and sensory characteristics of the beverage **for one month** at 6°C instead of two-three days as at the beginning of the project.. During the project ***Fermented Soy Beverage*** was **for the first time produced and marketed by a SME,** as described on a website appositely realised for consumers (www.sojo.cz). The new starters set up, both comprising one *Streptococcus thermophilus* strain and one *Bifidobacterium* as *FSB* biotum

supplement, allowed to produce *FSB* samples in which both ***Bifidobacterium*** and ***Str. thermophilus*** strains **maintained their population for one month** of cold storage, and a well perceived sensory profile.
Furthermore, the finding of high content of resistant starches in *Boza*, polyunsaturated fatty acids in *FSB* and more than 90% of L(+) lactic acid in both these beverages, provide preliminary information about potential beneficial effects on human health.
Finally, all fermented **beverages** produced **met the EU regulation concerning food hygiene** and safety, requirement essential for introducing these drinks into new markets.

1.2 Introduction

Fermentation is a biological process causing biochemical modification in raw foods such as milk, meat, and vegetables. In fermented foods properties as taste, flavour, texture, and nutritional value usually increase. In addition, fermented foods have an extended shelf-life in comparison with their raw materials.
In FERBEV project the following fermented beverages, based on plant raw materials or milk, were processed:

- *Boza*: a traditional Turkish cereal-based fermented beverage from a natural starter
- *Ayran*: a traditional Turkish yoghurt type product consumed as a drink after the addition of water and salt
- *Matsony*: a traditional Georgian fermented milk
- *Fermented Soy Beverage*: a new fermented drink based on soy preparation, developed in Poland.

Boza is a traditional cereal-based fermented cold drink with a slightly acidic sweet flavour. This beverage originated in Mesopotamia 8000-9000 years ago. *Boza* is mainly made from hulled millet, which is boiled in water and then poured into broad shallow pans. When cool, the mixture is sieved, and water and sugar are added. *Boza* is produced in most Turkish regions as well as in Bulgaria, Albania and Romania. Different cereals (wheat, millet and rye) can be used for *Boza* production, and natural mixtures of yeasts and lactic acid bacteria cause fermentation. Interactions between microorganisms are uncontrolled during the process, which leads to a variable product quality and a short shelf-life. As the shelf-life of *Boza* is limited to one or two weeks, it cannot takes summer temperatures and if it is not stored under refrigeration conditions, the product spoils and becomes inedible after a few days. Traditionally, the consumption of *Boza* is limited to the winter months, so production is generally carried out from September to March.

Ayran is a yoghurt drink produced in Turkey; this beverage is traditionally manufactured by adding water to yoghurt at a level of 30–50% and salt at a maximum level of 1%. *Ayran* is distinct from other fermented milk beverages, being a yoghurt drink with salt and without any fruit flavouring. The quality of natural *Ayran* deteriorates during storage due to acidity development and serum separation limiting the shelf-life to 10–15 days at refrigeration temperatures. Optimum consistency for good mouthfeel and no serum separation during storage are desired quality criteria in *Ayran*.

Matsony is a traditional Georgian dairy product similar to yoghurt inoculated with 3-5% of previous day batch of product and fermented for 5-7 hours. The dairy acid of *Matsony*, apart from fermentation products, also contains products of alcoholic fermentation such as carbon dioxide. Due to the utilization of commercial yoghurt starter cultures, the biodiversity of natural microflora is decreasing and consequently the traditional taste of *Matsony* is always closer to the taste of yoghurt. It has to be noted that during milk pasteurization whey protein can be added, giving the product a gentle consistence that in turn improves its flavouring. Due to uncontrolled post-acidification process, the shelf-life of *Matsony* is limited to 5-7 days at refrigeration temperatures.

Fermented Soy Beverage (*FSB*) is a new product developed at laboratory scale by Polish researchers in order to provide a pro-healthy drink to citizens replacing part of animal based foods. Laboratory results needed to be confirmed and transferred to real production conditions. In this way the exploitable benefits for human health can be spread among people.

1.3 Project objectives

The guiding principles were:

1) **to obtain an understanding** of the chemical, nutritional and microbiological characteristics of these fermented beverages (two milk-based and two vegetable-based drinks) that are still poorly known;
2) **to modernize the existing fixtures and fittings and/or plan and install new lines**;
3) **to standardize production** of beverages and **extend their shelf-life** by the use of selected autochthonous starters;
4) **to acquire preliminary information about the potential beneficial effects on human health** of the microorganisms and chemical components present in these fermented beverages.

In order to develop and design the innovative FERBEV process, several major innovations/advantages were considered:

- better understanding of the beverage fermentation process
- safeguarding microbial biodiversity
- assessment of adequate beverage processing steps
- innovative approaches to the production process of pro-healthy/probiotic foods
- production of beverages under more hygienic conditions and with a longer shelf-life
- export of these products all over the world thus gaining new customers
- growth on the European market of the new brand of fermented foods to which cereal and soy-based fermented beverages belong.

1.4 Contractors involved

The project has initially involved 14 participants: eight SMEs (beverage manufacturers, technologies and plant suppliers) and six RTD public bodies and Institutes from across Europe (located in Italy, Turkey, Poland, Georgia, United Kingdom and Czech Republic). A SME partner (Georgian Products Ltd, Georgia) abandoned the project at the end of the first year and the related activities were shared among the others contractors.
The consortium expertises were well balanced in the relevant technologies, existing structures and facilities, marked needs knowledge, etc., brought together to create new opportunities and benefits. More than seventy peoples among researchers, entrepreneurs, technicians and students have been assigned to 8 work packages in charge for the activities in the overall work plan.
Table 1 lists all participants and their role in the FERBEV project

Table 1: Ferbev Partnership

Partner		Short Name	Country	Role
(1) Consiglio Nazionale delle Ricerche, Istituto di Scienze delle Produzioni Alimentari, Bari	RTD Performer	CNR-ISPA	Italy	Coordinator and leader of WP 2, 6, 7 and 8
(2) TUBITAK Marmara Arastirma Merkezi, Gebze-Kocaeli	RTD Performer	TUBITAK	Turkey	WP1 Leader
(3) East West Economic Network-Georgia, Tbilisi	RTD Performer	EWEN	Georgia	WP5 Leader
(4) Instytut Biotechnologii Przemysłu Rolno-Spożywczego, Warsaw	RTD Performer	IAFB	Poland	WP6 Leader
(5) Università Cattolica del Sacro Cuore, Milan	RTD Performer	UNICATT	Italy	WP4 Leader
(6) The University of Liverpool, Dept. of Electrical Eng. & Electronics, Liverpool	RTD Performer	UOL	United Kingdom	WP3 Leader
(7) Deniz Tic A.S., Ankara yolu-Bursa	SME	DENIZ TIC	Turkey	*Boza* producer
(8) Aygin Süt Ve Gida Mamulleri Sanayi Ve Ticaret Anonim Şirketi, Karatay, Konya	SME	AYGIN SÜT	Turkey	*Ayran* producer
(9) Intermak Makine Imalat Ithalat, A.S., Karatay, Konya	SME	INTERMAK	Turkey	Producer of starters for *Ayran* and *Boza*
(10) Amaltea Joint-Stock Company, Tbilisi	SME	AMALTEA	Georgia	*Matsony* producer
(11) Plastcom A.S., Prague	SME	PLASTCOM	Czech Republic	*FSB* producer
(12) SACCO S.R.L., Cadorago, Como	SME	SACCO	Italy	Producer of starters for *Matsony* and *FSB*
(13) Milk Project Food Engineering S.R.L., Merone, Como	SME	MPFE	Italy	Machine producer

1.5 Work performed and end results

The project approach overlaps eight integrated work-packages: WP1-WP6 committed to achieve the research plan objectives, WP7 devoted to innovation related actions, and WP8 to consortium management activities.

WP1: Physicochemical, microbial and sensory characterisation of fermented beverages

The project first objective (WP1) was to define the gross chemical and nutritional characteristics and microbial populations of fermented beverages. For each beverage a nutritional table was delivered (**M1**) and at least one hundred isolates belonging to lactobacilli, gram positive cocci and yeasts were harvested from each beverage (**M2**). The average values of main nutrients for each drink are reported in Table 2.

Count and isolation of microbial isolates were carried out following traditional plating technique by using some selective media in order to enumerate and isolate different microbial populations occurring together in the same sample.
As concerns isolates from traditional beverages, about 200 bacterial colonies and 50 yeasts were isolated from *Boza* samples, more than 230 microbial colonies, including yeasts, were obtained from dominant populations of *Ayran* samples and 250 isolates were harvested from artisanal *Matsony* samples.
Fermented Soy Beverage strains were available from IAFB culture collection.

Table 2: Gross chemical composition of traditional fermented beverages and innovative *Fermented Soy Beverage (FSB).*

	BOZA			***AYRAN***			***MATSONYI***			***FSB***		
	Content	**Energy value[1]**		**Content**	**Energy value[1]**		**Content**	**Energy value[1]**		**Content**	**Energy value[1]**	
	% (w/w)	kcal	kJ	% (w/w)	kcal	kJ	% (w/w)	kcal	kJ	% (w/w)	kcal	kJ
Proteins	0,5	2,0	8,5	2,7	10,7	46,7	4,9	19,6	83,3	3,4	13,6	57,8
Fats	0,2	2,3	9,4	2,3	20,6	84,7	1,5	13,2	54,4	1,4	12,6	51,8
Total carbohydrates	13,0	34,2	145,5	3,0	12,1	51,4	5,3	21,3	90,4	13,3	32,6	138,5
Available carbohydrates	8,6	34,2	145,5	3,0	12,1	51,4	5,3	21,3	90,4	8,1	32,6	138,5
Unavailable carbohydrates[2]	4,4									5,2		
Ash	0,1			1,1			1,0			0,4		
Total solids	13,8			9,1			12,7			18,0		
Energy value of drink[2]		38,5	163,4		43,4	182,8		54,1	228,1		58,8	248,1

[1] for 100 g of fresh beverage.
[2] glucans, fructans, raffinose, stachyose.

WP2: Isolation, molecular typing, identification and evaluation of technological properties of microbial strains dominant in the fermented beverages

Differentiation and identification of collected strains from different steps of manufacture processes of each fermented beverage were performed in **WP2**. Within this WP the characterization of microbial metabolisms involved in fermentation or spoilage of fermented beverages was also evaluated. At the end of the work at least 10 different microbial strains, endowed with one or more technological features from each beverage, had to be selected and eventually used as selected starter in the following research activities (**M3**).
Several molecular techniques were used to identify and cluster microbial isolates picked up from beverage samples. DNA was usually extracted by using commercial kits. Isolates were clustered on the basis of their RAPD (Random Amplified Polymorphic DNA) PCR fingerprints obtained by using different random primers, and biotypes were identified by sequence analysis comparison of rRNA gene small subunit and, in some cases, by species-specific PCR.
To achieve a thorough picture of complex microbial populations occurring in beverages PCR DGGE (Denaturing Gradient Gel Electrophoresis) of V3 or V6-V8 16S rDNA fragments either directly extracted from samples or from cells harvested from countable agar plates (bulk) were carried out.
The metabolisms investigated depended on interaction between isolated strains and beverages. So, lactobacilli and streptococci were evaluated for acidification curve in milk, proteolytic activity and exopolysaccharide production when isolated from milk-based beverages, whereas as the main nutritional substances present in *Boza* are carbohydrates, isolates from this beverage were characterized for their ability to ferment different carbon sources and produce CO_2.
Other biotechnological characterizations such as antagonistic activity against pathogenic microorganisms and short chain fatty acid production were evaluated for a large part of the isolated

strains. For some particular groups of microorganisms, a more in depth evaluation was performed such as resistance to heating and freezing for *FSB* strains or ethanol production, invertase activity, and L-phenylalanine metabolism for yeasts from *Boza*.
The research activities carried out on beverage samples allowed to collect more than seven hundred microbial isolates from beverages. After molecular biotyping and taxonomic identification a FERBEV microbial culture collection was built up with the contribution of strains from IAFB, UNICATT and SACCO microbial collection. More than 200 bacterial and yeast strains were harvested from FERBEV beverages.

WP3 Studies on preservation and shelf-life extension of Boza, Ayran, Matsony and Fermented Soy Beverage

The shelf-life period of traditional beverages is usually short and hampers to enlarge the market of sales. For this reason **WP3** aimed to extend fermented beverages shelf-life also by using new methods such as UV and Ozone treatment. In this way it would be feasible to extend the shelf-life of milk and soy based beverages to more than one month and that of *Boza* until six months (**M5**).
UV treatments, mainly when serially applied, were more efficient in lowering microbial loads of *Boza* than those of milk based beverages. *Boza* and *Ayran* were also directly ozoneated with a good inhibition of microbial growth even though slight changes in this procedure need to be introduced in the daily production. Based on these outcomes ozone treatments planned for *Matsony* were not carried out.
Anyway, *Ayran* and *Matsony*, produced after improving hygienic conditions, were stable up to one month following correct production and storage procedures.
Furthermore, *Boza* heat treatment showed microbial killing rates similar to those obtained by ozone treatment without changing its viscosity whereas drying experiments lowered *Boza* microbial cell counts without completely killing lactic acid bacteria. Depending on treatment applied, *Boza* shelf-life was extended from two up to six months. The best results were obtained storing samples at 4°C.
As *Fermented Soy Beverage* is a drink enriched in probiotic microorganisms, no physical treatments were carried out on fresh *FSB* since they reduce microbial viable cell counts. In accordance with similar fermented beverages already present on the market *FSB* shelf-life period is of 30 days at 4°C.

WP4: Pilot scale production of fermented beverages using selected microbial strains prepared as single- or multiple-strain culture

WP4 aimed to produce fermented beverages using different mixtures of microbial strains previously selected for interesting technological properties and resistance to freeze drying process. At least two selected autochthonous starters for each traditional fermented beverage were realised. As concerns *Fermented Soy Beverage*, two strain sets, each comprising one *Streptococcus thermophilus* strain as starter and one *Bifidobacterium* strain, as *FSB* biotum supplement, were formulated (**M6**).
Acidity and pH values during shelf-life such as lactic and acetic acid contents, and ethanol release and accumulation were evaluated in all *Boza* samples manufactured by using the selected autochthonous starters. Changes in viscosity values, another important quality parameter, were also analysed during cold storage. Furthermore, *Streptococcus thermophilus* strains were also characterized for their sensitivity to bacteriophages. All combinations of lactic acid rods and cocci were evaluated for their acidification curves. Proteolysis in milk was also analysed for some microbial combinations.
The main chemical parameter checked in all *FSB* samples was the L(+) lactic acid content, considered important for *FSB* digestibility.
As concerns *Boza*, 13 autochthonous starter mixtures, including bacteria and yeasts, were assayed in laboratory test productions. The results of these tests led to obtain two selected starters to be used in pilot scale productions.

Four starters were tested in laboratory scale *Ayran* productions. The evaluation of microbiological and chemical analyses allowed the selection of two new autochthonous starters for pilot plant trials.
Seven different microbial mixtures were tested under laboratory scale conditions for *Matsony* production. Two of them were the most promising to be used in pilot scale production.
Fermented Soy Beverage was obtained, during WP4 research activities, by using three different microbial starters. One of these versions was split in two variants thus two starters were used for setting up the microbial part of process prototype.

WP5: Plan and creation of new pilot scale fittings and fixtures for production lines of fermented beverages

The suitability of starter strains studied under laboratory conditions was verified by using a new equipment (**WP5**). This new plant was designed, obtained, installed and set in production with the goal to set up at least three productions for each beverage (**M7**). The pilot plant was used to manufacture the three traditional fermented beverages by changing the flows of material. In particular, it was successfully used for manufacturing *Ayran* and *Matsony*, realizing five and six productions, respectively. For these beverages the plant is ready to be employed for daily fermented milk productions carried out in small size enterprises and it can also be up-scaled for larger productions.
Pilot plant also worked successfully for manufacturing *Fermented Soy Beverage*: all steps needed to pasteurize and ferment soy ingredients were performed (3 workings). Tests carried out for *Boza* manufacturing (3 productions) showed that a full working of plant can be reached replacing the existing vibrating filter with a centrifugal separator.
Microbial kinetics of all these pilot plant scale productions were followed by applying traditional and molecular techniques.

WP6: Maintenance studies of chemical, microbial, and sensory properties of beverages obtained utilizing project results

As final proof of practical transfer of research results to SME daily practice, the WP6 aimed to evaluate the improvement and standardization of beverage processing with particular regard to chemical, microbiological and sensory features of fermented drinks. These products, manufactured by utilising research activity results, have to meet the European food hygienic regulations (**M8**) .
Seventeen beverage productions were obtained.
Traditional beverages manufactured by using freeze-dried autochthonous starters and new equipment specifically set up for the project met the European Regulations on microbiological criteria for foodstuffs without loosing their original organoleptic characteristics.
In addition, *Boza* and *Matsony* shelf-life was considerably extended giving to SME producers the possibility to enlarge their markets.
An innovative *Fermented Soy Beverage* was set up by studying and testing all phases of production from recipe to microbial survival during 30 days of cold storage. The joint efforts of SME producers and RTD performers were rewarded by the entry of *FSB* into the market, as described on a website appositely realised for consumers (www.sojo.cz).
The finding of high content of resistant starches in *Boza*, polyunsaturated fatty acids in *FSB* and more than 90% of L(+) lactic acid in both these beverages, provided preliminary information about potential beneficial effects on human health (**M9**).

1.6 Major achievements

Resistant starches in Boza

Boza is a fermented cereal-based fermented cold drink with a slightly acid sweet flavour. *Boza* is mainly made from corn, wheat and millet, that are boiled in water, and then poured into broad shallow pans. When cool, the mixture is sieved, water and sugar added, and stored in fridge. Fermentation process of cereal-based beverages enhances the formation of resistant starch (RS), that, escaping digestion in the small intestine, induces several valuable physiological effects (SCFA production, reduction in faecal pH and plasma lipids, faecal moisture increase, lowering of glycaemic response to a meal, viable bifidobacteria cell increase).

FERBEV project research activities showed that *Boza* can be considered a natural source rich in resistant starches with percentages ranging from 5.89 to 11.08%. To our knowledge this is the first report on the finding of resistant starch in this ancient Turkish beverage and this finding is an interesting scientific evidence supporting potential beneficial effects of *Boza* on human health.

In accordance with the EC directive 2008/100/EC, concerning the nutritional labelling for foodstuffs, the *Boza* producer DENIZ TIC can include the RS content of this beverage under the term "Dietary Fibre" displayed on the nutrition information panel of the package of *Boza*. In accordance with the EC Regulation n. 1924/2006, the nutritional claim "High fibre" can be also marked on the *Boza* package with the defined formulation considered above. Furthermore, RS3 from *Boza* can be used for fortifying foodstuffs and drinks up to 6% like many other naturally processed RS commercially available and used for the same purpose.

DENIZ TIC that produces *Boza* is interested to enter into commercial agreement with distributors of the European market. The kind of cereals used, the amount of each cereal mixed and process parameters useful for obtaining *Boza* at high amounts of resistant starches are covered by IPR, described in the Title V, Art. 2.4 of the Consortium Agreement.

Nutritional characteristics of Fermented Soy Beverage

Fermented Soy Beverage, as produced following IAFB's recipe, was analysed for fatty acid composition finding that more than 80% of total fatty acids (about 3.8 g/100 ml of beverage) are constituted by unsaturated fatty acids, of which more than 60% are polyunsaturated fatty acids. Based on the Scientific Panel on Dietetic Products, Nutrition and Allergies of the European Food Safety Authority (The EFSA Journal (2005) 253, 1-29), *FSB* can be considered as "High unsaturated fat" or eventually "High polyunsaturated fat".

These results, including the addition of 60 mg/100 ml of omega-3 fatty acids, decided by *FSB* producer PLASTCOM, indicate that *FSB* is a valid snack for people affected by cardiovascular diseases.

Fermentation of milk usually produces racemic lactic acid: D-lactic acid cannot be digested in the human body, gathering and causing acidosis differently from L(+) lactic acid that is used by human body for energy purpose. Depending on the starter used, *FSB* samples presented 100% of L(+) lactic acid and also thanks to the high amounts of healthy viable bifidobacteria present, *FSB* can be considered an appropriate beverage for children.

As the beverage is already on the market, PLASTCOM is searching for selling agents across European Community.

Ingredients, process parameters, microbial starter and probiotic supplement used for *FSB* are covered by IPR, as described in the Title V, Art. 2.4 of the Consortium Agreement.

Extension of fermented beverage shelf-life

Two new devices, an UV apparatus and an Ozone device, set up exploiting the pre-existing UV bulb, were assembled and applied for extending the shelf-life of beverages.

UV treatment was efficient in lowering microbial loads of *Boza*, *Ayran* and *Matsony*, mainly when serially applied. In particular, serial UV treatments were highly efficient in *Boza* and less in milk-based beverages. However, the need of diluting beverages before UV treatment indicates that the UV device can be used to extend the shelf-life of beverages with a lower light absorption coefficient.

On the other side, beverage samples of *Boza* and *Ayran* were directly ozoneated without the necessity of diluting drinks; however, treatments caused an appreciable change in traditional *Boza* taste and foam production in *Ayran*.

Depending on the beverage, different methods for improving shelf-life can be applied.

As concerns UV and Ozone devices, they resulted very effective in killing microorganisms mainly when applied to not viscous or fat beverages. These devices are effective for liquid no-fat beverages and need only to be scaled up.

Depending on treatments applied, *Boza* shelf-life was extended from two up to six months in comparison with commercial *Boza*, whose shelf-life is not longer than two weeks

RTD performers intend to carry out further researches in order to better define process parameters for improving foodstuffs shelf-life.

FERBEV microbial culture collection

About seven hundred bacterial and yeast isolates were harvested from traditional beverages, milk and cereals in the course of the project. The application of molecular techniques such as Random Amplified Polymorphic DNA RAPD)- PCR made it feasible to cluster isolates into more than two hundred strains whereas rDNA sequence analyses and species-specific PCR allowed to ascertain that strains belonged mainly to *Streptococcus thermophilus*, *Leuconostoc lactis*, *Lactobacillus delbrueckii,* and *Enterococcus faecium*. As concerns yeasts, 25 different strains, mainly belonging to *Pichia fermentans* species, were isolated from beverages. All strains were stored in the FERBEV microbial collection.

The main source of strains for FERBEV microbial collection was *Boza,* followed by *Ayran,* and *Matsony*. This collection also includes some strains coming from IAFB, UNICATT and SACCO microbial collection.

The collection, stored in two different sites (TUBITAK and UNICATT), is available for all FERBEV partners and can be used by each partner to obtain new or improved fermented foods.

Furthermore, these microorganisms can be further studied in other research projects for applications different from those evaluated in FERBEV and raise interest in different stakeholders such as food industries and other research institutes, that can cooperate with FERBEV partners in order to exploit microorganisms for other applications.

Genetic characterization of enterococci from Boza

Enterococci are gram-positive bacteria of human and animal gastrointestinal tract origin. Studies on the microbiota of many Mediterranean typical foods indicated that enterococci play an important role in ripening contributing to their typical taste and flavour. In recent years, nosocomial infections due to enterococci with multiple-drug resistances and virulence factors have increased throughout the world. More than 30 bacterial colonies were isolated from *Boza* samples and identified as *Enterococcus faecium* (17 strains) and *Ent*. *durans* (4 strains). They were genetically characterized in order to evaluate their pathogenicity in *Boza* beverage. PCR screening for virulence determinants included: gelatinase gelE, virulence regulator fsr, cytolysin cylA, cylB, cylM, adhesion factors asa373, esp, efaA, agg, pheromones cad and PAI pathogenicity island. A surprisingly result was

that, differently from other sources such as milk and meat based foods, all *Boza* enterococci lacked known virulence factors suggesting that *Boza* cannot be considered as a vehicle of potential pathogenic enterococci strains.
This result opens a new field of research as large part of enterococci from other foods carry out one or more virulence factor.
Further researches will demonstrate whether strains from *Boza* can be used in food manufacturing replacing enterococci harbouring virulence factors.

Antagonistic activity of some FERBEV microorganisms
Sixty-four LAB strains, isolated from *Boza* and *Ayran* samples, were assayed for their antagonistic activity against three pathogenic bacteria, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*, usually found in milk and/or other fermented beverages. *E. coli* was the most inhibited strain, in particular by *Lactobacillus delbrueckii bulgaricus* strain 268, whereas *S. aureus* was the most resistant species strongly inhibited only by the *Lactococcus lactis* 52. Fourteen LAB strains reduced *B. cereus* growth from 75% to 100%.
Pichia fermentans strains were able to inhibit the growth of *Trichosporon cutaneum* DSM 70684, responsible for some skin diseases.
Results on antagonistic activity of bacteria used in *Fermentad Soy Beverage* showed that *Str. thermophilus* T_KM_3, developed its stronger antagonistic activity against *Cl. perfringens* KKP1206, whereas *Lb. plantarum* KKP384 and *Bifidobacterium breve* KN65 were active at different levels against all indicator strains.
These results open the possibility to identify new inhibitory substances or use some strains in order to improve safety of FERBEV beverages also in case of post contamination by pathogens.
The strains able to control pathogens can be applied in food industry. Further studies need to be performed in order to evaluate how inhibitory activity is expressed. Inhibiting strains should be further characterized and, on the basis of results, made available to food companies.

Proteolysis in milk based beverages
Proteolysis in yoghurt, accomplished by lactic acid bacteria proteinases and peptidases, can severely influence flavour and texture of the product as the enzymatic degradation of bovine caseins may lead to formation of biologically active peptides.
The peptides found, including those putatively with bitter taste, can contribute to overall typical flavour of salted yoghurt *Ayran*. The thorough proteolysis by some *Lb. delbrueckii* subsp. *bulgaricus* strains can support the growth of *Str. thermophilus* with a proto-cooperative mechanism standardizing the mixed starter culture.
The proteolytic pattern of *Str. thermophilus* strains in milk confirmed the results obtained by PCR protocols that showed the absence of the DNA fragment related to *prt*S gene (responsible for a cell envelope proteinase) in all *Str. thermophilus* strains isolated from *Ayran* and *Matsony*.
Different strain combinations produced different patterns of proteolysis of milk proteins. In this way it can be possible to drive, partially, the proteolysis of fermented milks.
The release of ACE inhibiting or opioid peptides can be performed. The presence of these peptides will lead the production of functional fermented milks.
Finally, microbial strain combinations should be further characterized for releasing functional peptides, and, on the basis of results, made available to food companies.

Set up of an in vitro *method for the selection of potential probiotic strains*
The necessity to isolate directly from fermented beverages potential probiotic strains without analyzing hundreds of colonies led us to set up an *in vitro* digestion protocol.

The application of this protocol to commercial probiotic fermented milks made it feasible to isolate only the probiotic strains mentioned in the label whereas other lactic acid bacteria, usually occurring in these yogurt-like fermented milks, were not recovered.
At the end of the experiments carried out on traditional fermented beverages, one strain of *Pichia fermentans* was recovered from *Boza* whereas one strain of *Lb*. *fermentum* and one of *Enterococcus faecium* were isolated from *Ayran* samples and one *Lb*. *plantarum* strain was isolated from *Matsony*.
The isolation of potentially probiotic strains is a promising result and some of these strains can be used as adjunct for manufacturing traditional beverages with beneficial effects on human health.
The method can be used, also by food industry, to isolate new potentially probiotic strains directly from many food samples.

2 Dissemination and use

WP7: Innovation related activities

WP7 of FERBEV work plan addressed all the activities aimed at dissemination, exploitation planning as well as the consortium recommendations and market approach and strategy. The innovation related activities foresaw two tasks: the first one concerned the dissemination of knowledge in scientific, consumer addressed and educative ways (**M10**) and the second one the IPR protection.
A high level of dissemination has been maintained throughout the project, ranging from many presentations and published articles on the project itself, to consultation with SMEs, consumers, etc. Additionally, partners undertook various dissemination activities at national level, including informal and formal meetings.

In order to immediately improve the Project visibility, a logo was designed and used in all the dissemination tools, ranking from the web site to fact sheet and brochures.
The first Dissemination task was the development of a dedicated Web page. A dedicated portal (http://www.ferbev.net/) was established early in the project, primarily to provide information related to the project and also used as work area and document repository for the partners. Main public information available:

- general description, which includes a short project presentations in different languages (Turkish, Georgian, Polish, Italian, Czech and English) for external users
- project objectives and partners
- project news and events; in this section visitor can find all available project news
- fermented beverages
- downloads area with the presented scientific posters at workshops and seminars, as well the publishable final activity report.

This section is complemented by deliverables D6-Dissemination of knowledge and Project presentation and D7-Web Site where details of the dissemination and community building activities carried out are given.
The second task of WP7, related to IPR protection, was developed, discussed, and general rules were approved during FERBEV meetings. In addition, some SMEs signed special contracts defining how managing the joint properties arising from the project. Detailed information are available on the FERBEV website (www.ferbev.net). Considering that the Intellectual Property Rights of the results belong exclusively to the SME participants, and in order to maximise the overall usage of the FERBEV achievements, the SMEs concerned agreed amongst themselves on the allocation and the terms of exercising the ownership according its business mutual competitive advantage.

3 Publishable results

Several promising and sometimes unexpected interesting results were obtained in the course of the project.

Novel microbial starters for *Boza*, *Ayran*, *Matsony* and *Fermented Soy Beverage* were set up in the course of the project research activities. *Boza* and *Fermented Soy Beverage* are produced fermenting cereal and soy proteins, respectively whereas *Ayran* and *Matsony* are milk based yogurt type drinks.

In order to obtain a good fermentation process and a palatable beverage, different microbial combinations were analysed and tested in pilot scale productions.

Boiled cereal mixtures were fermented with more than 10 different microbial starters with or without yeasts evaluating the better combinations able to produce a good *Boza* stable under cold storage conditions. At the end of the assays, two different autochthonous starters were obtained mixing homofermentative and heterofermentative lactic acid bacteria and a single yeast strain.

From *Ayran* and *Matsony* microbiota several bacterial strains belonging to homofermentative thermophilic lactic acid bacteria were tested in different combinations. On the basis of the study of autochthonous microorganisms occurring in traditional beverage samples and technological characteristics displayed by single strains, three starters were produced for these milk-based beverages able to ferment milk under standardized conditions; they improve beverage quality avoiding post-acidification occurring during fridge storage.

Fermented Soy Beverage was produced in different versions as pure or fruity drink. *FSB* was fermented by using several combinations of different lactic acid bacteria and adding, before cold storage, adequate amounts of bifidobacteria strains. Among all versions evaluated, the best combination was able to maintain a good taste and flavour during cold storage and high viable bifidobacteria cell counts.

3.1.1 Resistant starches in *Boza*

Result description	*Boza* is a fermented cereal-based beverage whose seeds are cooked in water, cooled, fermented. amended with sugar and stored in fridge. Cereals, used in different ratio for *Boza*, include corn, wheat. millet and rye; potatoes are also used. Fermentation process of cereal-based beverages enhances the formation of resistant starch (RS), which resists *in vitro* to the enzymatic hydrolysis and escapes digestion in the small intestine being hydrolysed in the large intestine. RS induces several valuable physiological effects (SCFA production, reduction in faecal pH and plasma lipids, increase in faecal moisture, lowering of glycaemic response to a meal, increase in viable bifidobacteria cells). Many naturally processed RS are commercially available and used for fortifying foodstuffs and drinks up to 6%. FERBEV project research activities demonstrated that *Boza* can be considered a natural source rich in resistant starches with percentages ranging from 5.89 to 11.08%. In accordance with these early results the resistant starch found in *Boza* samples are mostly considered to belong to RS3 type due to technological process conditions. To our knowledge this is the first report on the finding of resistant starch in this ancient Turkish beverage and this finding is an interesting scientific evidence supporting potential beneficial effects of *Boza* on human health.
Possible market application	In accordance with the EC directive 2008/100/EC, concerning the nutritional labelling for foodstuffs, the *Boza* producer DENIZ TIC can include the RS3 content under the term “Dietary Fibre” displaying in nutrition information panel of the package of *Boza* sample as considered above. In accordance with the EC Regulation n. 1924/2006, the nutritional claim “High fibre” can be marked on the *Boza* package with the defined formulation considered above. Furthermore, RS3 from *Boza* can be used for fortifying foodstuffs and drinks up to 6% as many other naturally processed RS commercially available and used for the same purpose.
Stage of development	Completed
Collaboration sought or	DENIZ TIC that produces *Boza* is interested in commercial agreement with distributors especially for the European market.

offered	
IPR	The kind of cereal used, the amount of each cereal mixed and process parameters useful for obtaining *Boza* at high amount of resistant starches are ownership of DENIZ TIC. DENIZ TIC. A.S. Oto Sans T Sanay S Tes 44.Blok, 16.Sokak No:47-49, Ankara Yolu Bursa - **TURKEY**
Contact details	Mr. Mustafa ÇEVIK Phone +90-224-3461103 Fax +90-224-3461104 e-mail info@ekinboza.com

3.1.2 Nutritional characteristics of *FSB*

Result description	*Fermented Soy Beverage*, as produced following IAFB's recipe, was analysed for fatty acid composition. In comparison with yogurt type beverage *FSB* was found to be reach in long chain fatty acid both saturated and unsaturated. More than 80% of total fatty acids (about 3.8 g/100 ml of beverage) are constituted by unsaturated fatty acids, of which more than 60% was constituted by polyunsaturated fatty acids. Based on the Scientific Panel on Dietetic Products, Nutrition and Allergies of the European Food Safety Authority (The EFSA Journal (2005) 253, 1-29), *FSB* can be considered as: "*High unsaturated fat*": as the amount of unsaturated fat is 70% of the total fat content in the product. and eventually "*High polyunsaturated fat*": as at least 45% of the fatty acids present in the product derive from polyunsaturated fat and saturated fat must not provide more than 10% of energy. As PLASTCOM added to original IAFB recipe omega-3 fatty acids, 60 mg/100 ml beverage, these data indicate that *FSB* can be a valid snack in particular for people affected by cardiovascular diseases also thanks to the absence of cholesterol. *FSB* was produced fermenting ingredients by using different lactic acid bacteria and adding different *Bifidobacterium* species. Fermentation of milk usually produces racemic lactic acid. The D-lactic acid cannot be digested in the human body gathering and causing acidosis differently from L(+) lactic acid that is used by human body for energy purpose. Some of FSB samples, depending on starter used, presented 100% of L(+) lactic acid and also thanks to the high amount of healthy viable bifidobacteria cells present, *FSB* can be considered an appropriate beverage for children.
Possible market application	The innovation is related to agrofood sector. The beverage is already on the market.
Stage of development	Completed
Collaboration sought or offered	PLASTCOM searches for selling agents across European Community
IPR	The ingredients, the process parameters, the microbial starter and the probiotic supplement used for *FSB* are covered by IPR described in the Title V, Art. 2.4 of the Consortium Agreement PLASTCOM A.S. U Sparty, 14 170 00 Prague - **CZECH REPUBLIC**
Contact details	Ing. Stepan TUMA Phone +420-602-683-641 Fax +420-233-372-477 e-mail stepan.tuma@firemni.cz

3.1.3 Extension of fermented beverage shelf-life

Result description	Two new devices, an UV apparatus and an Ozone device, set up exploiting the pre-existing UV bulb, were assembled and applied for extending the shelf-life of beverages. UV treatment was efficient in lowering microbial loads of *Boza*, *Ayran* and *Matsony*, mainly

	when serially applied. In particular, serial UV treatments were highly efficient in *Boza* and less in milk based beverages. However, the need of diluting beverages before UV treatment indicates that the UV device can be used to extend the shelf-life of beverages with a lower light absorption coefficient. On the other side, beverage samples of *Boza* and *Ayran* were directly ozoneated without the necessity of diluting drinks; however these treatments caused an appreciable change in traditional *Boza* taste and foam production in *Ayran*. Both UV and ozone treatments were not useful for *Ayran* and *Matsony* shelf-life extension but these fermented drinks are stable up to one month following correct production and storage procedures, as usual for fermented milk based beverages. A similar result was obtained for *FSB* whose shelf-life period is of 30 days at 4°C. In addition, *Boza* was treated with heat and drying. Heat treatment (65-75°C for 20 min) showed killing rates similar to those obtained by ozone treatment without changing its viscosity. Drying *Boza* experiments showed good results in lowering microbial cell counts without completely killing lactic acid bacteria, although rehydration of dried *Boza* showed some changes in its structure. Depending on treatments applied, *Boza* shelf-life was extended from two up to six months in comparison with commercial *Boza,* whose shelf-life is no longer than two weeks.
Possible market application	Depending on beverage, different methods for improving shelf-life can be applied. As concerns UV and Ozone devices, they resulted to be very effective in killing microorganisms, mainly when applied on not viscous or fat beverages. These devices can be simply scaled up for liquid no fat beverages,.
Stage of development	UV and Ozone devices are under study whereas pasteurizing and drying equipment are available on the market.
Collaboration sought or offered	SME owners of knowledge and RTD performers intend to carry out further researches in order to better define process parameters for improving foodstuffs shelf-life.
IPR	The microwave UV lamp facility to produce the UV germicidal wavelengths (256nm) and Ozone from air is a patent awarded to UOL.
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3.1.4 FERBEV microbial culture collection

Result description	About eight hundred isolates were harvested from traditional beverages, milk and cereals in the course of the project. The application of molecular techniques such as Random Amplified Polymorphic DNA RAPD)- PCR made it feasible to cluster isolates into more than two hundred strains whereas rDNA sequence analyses and species-specific PCR allowed to ascertain that strains belonged mainly to *Streptococcus thermophilus*, *Leuconostoc lactis*, *Lactobacillus delbrueckii,* and *Enterococcus faecium*. As concerns yeasts, 25 different strains, mainly belonging to *Pichia fermentans* species, were isolated from beverages. All strains were stored in the FERBEV microbial collection. The main source of strains for FERBEV microbial collection was *Boza,* followed by *Ayran,* and *Matsony*. This collection also includes some strains coming from IAFB, UNICATT and SACCO microbial collection. The collection, stored in two different sites, is available for all FERBEV partners and can be used by each partner to manufacture new or improved fermented foods.
Possible market application	These microorganisms can be further studied in other research projects or used for applications different from those evaluated in FERBEV with other companies.
Stage of development	Completed
Collaboration sought or offered	Strains present in the FERBEV microbial culture collection can be of interest on behalf of different kinds of partners. Food industries can use FERBEV strains for new or improved foods. Other Research Institutes can cooperate with FERBEV RTD partners in order to exploit

IPR	FERBEV microorganisms for other applications. In addition to IPR described in the Title V, Art. 2.4 of the Consortium Agreement, more specific agreement were signed among some partners as concerns starter strains. During the final meeting the managing of these strains and their use after the end of the project were established.
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3.1.5 Genetic characterization of enterococci from *Boza*

Result description	Enterococci are gram-positive bacteria of human and animal gastrointestinal tract origin. Studies on the microbiota of many Mediterranean typical foods indicate that enterococci play an important role in their ripening contributing to typical taste and flavour. In recent years, nosocomial infections due to enterococci with multiple-drug resistances and virulence factors have increased throughout the world. More than 30 bacterial colonies were isolated from *Boza* samples and identified as *Enterococcus faecium* (17 strains) and *Ent. durans* (4 strains). They were genetically characterized in order to evaluate their pathogenicity in *Boza* beverage. PCR screening for virulence determinants included: gelatinase gelE, virulence regulator fsr, cytolysin cylA, cylB, cylM, adhesion factors asa373, esp, efaA, agg, pheromones cad and PAI pathogenicity island. A surprisingly result was that, differently from other sources such as milk and meat based foods, all *Boza* enterococci lacked known virulence factors suggesting that *Boza* cannot be considered as a vehicle of potential pathogenic enterococci strains.
Possible market application	None
Stage of development	Under study
Collaboration sought or offered	This result opens a new field of research as large part of enterococci from other foods carry out one or more virulence factor. Further researches will demonstrate whether strains from *Boza* can be used in food manufacturing replacing enterococci harbouring virulence factors
Collaborator details	
IPR	SMEs allow RTD performers to use these results for research purposes.
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3.1.6 Inhibitory activity of some FERBEV microorganisms

Result description	Sixty-four LAB strains, isolated from *Boza* and *Ayran* samples, were assayed for their antagonistic activity against three pathogenic bacteria, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*, usually found in milk and/or other fermented beverages. *E. coli* was the most inhibited strain, in particular by *Lactobacillus delbrueckii bulgaricus* strain 268, whereas *S. aureus* was the most resistant species strongly inhibited only by the *Lactococcus lactis* 52. Fourteen LAB strains reduced *B. cereus* growth from 75% to 100%. *Pichia fermentans* strains were able to inhibit the growth of *Trichosporon cutaneum* DSM 70684, responsible for some skin diseases. Results on antagonistic activity of bacteria used in *Fermentad Soy Beverage* showed that *Str. thermophilus* T_KM_3, developed its stronger antagonistic activity against *Cl. perfringens* KKP1206, whereas *Lb. plantarum* KKP384 and *Bifidobacterium breve* KN65 were active at different levels against all indicator strains. These results open the possibility to identify new inhibitory substances or use some strains in order to improve safety of FERBEV beverages also in case of post contamination by pathogens.
Possible market application	Strains able to control pathogens can be applied in food industry. Further studies need to be performed in order to evaluate how inhibitory activity is expressed.
Stage of development	Under study
Collaboration sought or offered	Inhibiting strains should be further characterized in cooperation with other research institutes, and, on the basis of results, then used by food companies.
IPR	SMEs allow RTD performers to use these results for research purposes.
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3.1.7 Proteolysis in milk based beverages

Result description	Proteolysis in yoghurt accomplished by proteinases and peptidases by lactic acid bacteria can severely influence the flavour and texture of this product like the enzymatic degradation of bovine caseins may lead formation of biologically active peptides. A total of 14 strains (3 *Str. thermophilus*, 5 *Lb. bulgaricus*, 1 *Lb. helveticus*, 1 *Lb. paracasei* and 4 *Lb. fermentum*), previously isolated from *Ayran* samples, were analysed for their capacity to release peptides after fermentation and cold storage (21 days). Among strains analysed, *Lb. delbrueckii* subsp. *bulgaricus* milk cultures displayed more

peaks (23 – 30) in pH 4.6 water-soluble extracts than those produced by *Str. thermophilus* and *Lb. fermentum* strains under the same conditions (11-16 and 9 – 16 peaks, respectively).
The proteolysis pattern of these latter strains did not differ from those reported on not inoculated milk, showing only 8 - 12 peaks, even after 3 weeks of cold storage. On the contrary, the RP-HPLC profiles derived from the stored sour milk, fermented with *Lb. delbrueckii* subsp. *bulgaricus* or *Lb. paracasei,* strains were different from the patterns of the same strains immediately after fermentation.
The peptides found, including those putatively with bitter taste, can contribute to overall typical flavour of salted yoghurt *Ayran*. The thorough proteolysis by some *Lb. delbrueckii* subsp. *bulgaricus* strains can support the growth of *Str. thermophilus* with a proto-cooperative mechanism standardizing a possible mixed starter culture.
The proteolytic pattern of *Str. thermophilus* strains in milk confirmed the result obtained by PCR protocols that showed the absence of the DNA fragment related to *prt*S gene (responsible for a cell envelope proteinase) in all *Str. thermophilus* strains isolated from *Ayran* and *Matsony*.

Possible market application	Different strain combinations produced different patterns of proteolysis of milk proteins. In this way it can be possible to drive, partially, proteolysis of fermented milks. The releasing of ACE inhibiting or opioid peptides can be performed. The presence of these peptides will lead to the production of functional fermented milks.
Stage of development	Ongoing
Collaboration sought or offered	Microbial strains combinations should be further characterized in cooperation with other research institutes for releasing functional peptides, and, on the basis of results, then used by food companies.
IPR	SMEs allow RTD performers to use these results for research purposes.
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3.1.8 Set up of an *in vitro* method for the selection of potential probiotic strains

Result description	The necessity to isolate directly from fermented beverages potential probiotic strains without analyzing hundreds of colonies, led us to set up an *in vitro* digestion protocol. The application of this protocol to commercial probiotic fermented milks made it feasible to isolate only probiotic strains mentioned in the label whereas other lactic acid bacteria, usually occurring in these yogurt-like fermented milks, were not recovered. At the end of experiments one strain of *Pichia fermentans* was recovered from *Boza* whereas one strain of *Lb. fermentum* and *Enterococcus faecium* were isolated from *Ayran* samples and one *Lb. plantarum* strain was isolated from *Matsony*. The isolation of potentially probiotic strains is a promising result and some of these strains can be used as adjunct for realizing traditional beverages with beneficial effects on human health.
Possible market application	The method can be used to directly isolate new potentially probiotic strains from many natural samples.
Stage of development	Completed
Collaboration sought or	Food industry

offered IPR	SMEs allow RTD performers to use these results for research purposes.
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