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DEVELOPMENT OF A FUNCTIONAL DAIRY FOOD ENRICHED WITH SPIRULINA (*ARTHROSPIRA PLATENSIS*)

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1. INTRODUCTION AND AIMS

Because of the changing dietary habits of the general public, functional foods are products of interest to many people. Consumers would need to ingest considerably less medicine and artificially produced vitamin and mineral supplements if fermented milks were enriched with vitamins, proteins, essential fatty acids, and trace elements of natural origin. A simple way of attaining this goal is the use of cyanobacteria in the manufacture of cultured dairy foods.

Spirulina is the dried biomass of *Arthrospira (A.) platensis*, which is a planktonic cyanobacterium that forms massive populations in tropical and subtropical water bodies characterized by high levels of carbonate and bicarbonate and high pH (up to 11). The green colored Spirulina, which tastes like grass, is a valuable food supplement that has a wide range of beneficial nutritional effects. It typically contains 3 to 7% moisture, 53 to 63% protein, 4 to 6% lipids, 17 to 25% carbohydrate, 8 to 13% ash, 8 to 10% fiber, 1 to 1.5% chlorophyll-*a*, and a wide range of vitamins.

The objectives of this work included:

1. Checking the microbiological quality of the powdered Spirulina biomass.
2. Testing the influence of the Spirulina biomass on acid production by various strains of *Lactococcus* (*Lc.*) *lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, and *Leuconostoc* (*Ln.*) *mesenteroides* in milk.
    a. Determining the optimum concentration of the cyanobacterial biomass in terms of sensory properties and costs.
    b. Finding *Lactococcus* strains whose acid production can be stimulated to a large degree by addition of the Spirulina biomass.

   c. Monitoring the changes in viable cell counts of lactic acid bacteria (LAB) indicated in point *b.* during the fermentation process.

3. Screening Spirulina extracts for inhibitory/stimulatory effects on foodborne pathogens and spoilage microorganisms by using the agar diffusion assay.
4. Developing production technology for a functional fermented milk manufactured with the LAB selected.
5. Running storage trials to determine the effect of the Spirulina biomass on viability of lactococci in the refrigerated product.

2. MATERIALS AND METHODS

The experiments were carried out in the accredited Microbiological Laboratory of the Institute of Food Science at the University of West Hungary.

2.1. Checking the microbiological quality of the powdered Spirulina biomass

The microbiological properties of commercial Spirulina powders were tested according to recommendations by the European Pharmacopoeia. The following microbial groups or species were enumerated: aerobic mesophilic microorganisms, yeasts, molds, enterobacteria, *Escherichia coli*, and coagulase-positive staphylococci. Presence/absence tests for detection of *Salmonella* spp. were also done.

2.2. Monitoring the changes in acid production and viable counts of mesophilic lactic acid bacteria grown in milk

Milk samples enriched with Spirulina at different concentrations (*i.e.*, 0%, 0,3%, 0,5% or 0,8%) were inoculated at the rate of 1% with the mesophilic LAB strains to be tested. Incubations were performed in a water bath set at 30°C. The pH value of three replicate samples from all treatments was measured at regular intervals (*i.e.*, every 2 h) with an HI 8521 pH-meter standardized with pH 4.01 and 7.01 standard buffer solutions. The experiments were repeated twice.

To monitor the changes in viable cell numbers of the lactococci strains selected, microbial counts of the samples were enumerated at h 0, h 6, and h

12 of the fermentation process in M17 agar using the pour-plate technique. The LAB strains used in the trials are shown in **Table 1**.

Table 1 List of lactic acid bacteria strains and cultures used in the trials

Lactic acid bacteria	**Designation of strain/culture**			
	BCCM*	**NCAIM****	**MTKI*****	**ATCC******
Lactococcus lactis subsp. *lactis*			Ha-2	
	LMG 8522	B.2125		
	LMG 9451	B.2128		
Lactococcus lactis subsp. *lactis* var. *diacetylactis*			W-24	
	LMG 7931	B.2122		11007
	LMG 7949	B.2123		20661
	LMG 9441	B.2126		13675
	LMG 9444	B.2127		
Lactococcus lactis subsp. *cremoris*	LMG 7951	B.2124		14365
	LMG 6897			19257
Leuconostoc mesenteroides subsp. *cremoris*	LMG 6909	B.2120		19254
Leuconostoc mesenteroides subsp. *dextranicum*		B.1658		19255
Lactococcus lactis subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis* var. *diacetylactis, Leuconostoc spp.*			CHN-22	
Lactococcus lactis subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis* var. *diacetylactis, Leuconostoc spp. Streptococcus termophilus*			XPL-1	

* Belgian Co-ordinated Collection of Microorganisms

** National Collection of Agricultural and Industrial Microorganisms

*** Hungarian Dairy Research Institute Inc. / Chr. Hansen

**** American Type Culture Collection

2.3. Determining the antimicrobial properties of the Spirulina biomass

The antimicrobial effects of various aqueous extracts from the Spirulina biomass on a total of 33 bacteria (including 18 Gram-positives and 15 Gram-negatives), 11 filamentous fungi, and 4 yeast species were tested using the agar diffusion assay. The substances screened included:

- Aqueous extract produced by 10 times dilution of the Spirulina powder (**A**);
- Supernatant obtained after centrifugation of **A** at 5000 rpm for 59 min (**C**);
- Extract from ultrasonic treatment of **A** at 130 W 60 sec (**U1**);
- Supernatant obtained by centrifugation of **U1** at 5000 rpm for 59 min (**U1C**).

The agar plates inoculated separately with the test organisms were incubated at the required preset temperatures for 24 h to 48 h. If present, the inhibitory substance(s) diffused from wells into the agar and resulted in an inhibition zone around the wells. The size of the inhibition zones accurately indicated the effectiveness of the active compound(s) against the organism tested.

2.4. Developing a functional fermented milk manufactured with mesophilic lactic acid bacteria and Spirulina biomass

As part of the product development process, three ranking tests were performed by 5, 11, and 12 sensory panelists, respectively, in an attempt to optimize the organoleptic properties of the final product. The samples were ranked according to the intensity of their sensory properties, with overall taste being the main ranking parameter.

2.5. Influence of the Spirulina biomass on mesophilic lactic acid bacteria during refrigerated storage of the model product

The Spirulina-enriched and control fermented milks used in the storage trials were manufactured at the pilot plant of the Hungarian Dairy Research Institute (Mosonmagyaróvár, Hungary). Antibiotic-free raw milk containing (per dm^3) 36.5 g of fat, 31.5 g of protein, 47 g of lactose, and 7 g of ash served as raw material. It was heated to 90°C and held for 10 min. In the case of the cyanobacterial product, Spirulina was added to the heat-treated milk cooled to 70°C. Plain milk and Spirulina-fortified milk were homogenized at a pressure of 18 MPa (180 bar) in a high pressure homogenizer. They were cooled to 30°C, and were inoculated with the starter culture selected by the sensory panelists. Incubation took approximately 10 h at 30°C. Sucrose at 10% and flavoring substances at 1.5% were then added during stirring at pH 4.7. Thereafter, the Spirulina-enriched and control fermented milks were both filled into 21 sterile, tightly capped centrifuge tubes (30 cm^3). The products were stored in a refrigerator at 4°C. Three cyanobacterial and three control samples were taken at weekly intervals, and their lactococci counts were enumerated. The experimental program was repeated twice.

3. RESULTS

3.1. Microbiological quality of the Spirulina biomass

The results of microbiological examinations revealed that the hygienic quality of the Spirulina biomass was good but not perfect. Although none of the samples tested contained enterobacteria, yeasts, or coagulase-positive staphylococci, the total plate counts of two products were close to the maximum allowable level of 10^5 CFU/g set by the European Pharmacopoeia. Mold counts were low in all of the samples tested.

3.2. Changes in acid production and viable counts of mesophilic lactic acid bacteria grown in milk

3.2.1. Optimum concentration of the Spirulina biomass

Spirulina levels capable of effectively stimulating acid production of lactococci were determined using *Lc. lactis* subsp. *lactis* Ha-2 and *Lc. lactis* subsp. *cremoris* W-24. The cyanobacterial biomass, used at 0.1% to 0.8%, was found to significantly increase ($P < 0.05$) the rate of acid development by lactococci between h 6 and h 12 of the fermentation process. For this reason and because of organoleptic and economic considerations, further trials were run with Spirulina use at 0.3% (i.e., 3 g/dm^3).

3.2.2. *Effect of the Spirulina biomass on single strains of mesophilic lactic acid bacteria*

The effects of 0.3% Spirulina on acid development by two strains of *Lc. lactis* subsp. *lactis*, two strains of *Lc. lactis* subsp. *cremoris*, four strains of *Lc. lactis* subsp. *lactis* var. *diacetylactis*, one strain of *Ln. mesenteroides* subsp. *cremoris*, and one strain of *Ln. mesenteroides* subsp. *dextranicum* were monitored during fermentation. The results are shown in **Table 2**. Negative numbers mean that Spirulina-enriched samples had higher pH than controls, whereas positive numbers indicate that the pH of cyanobacterial samples was lower than that of controls.

Means of the initial pH values (at h 0) of Spirulina-enriched samples were higher than those of controls because the cyanobacterial biomass is of alkaline character (an aqueous solution containing 3 g/dm^3 Spirulina has a pH of 9.9) and it also possesses considerable buffering capacity.

Table 2 Influence of 0.3% Spirulina biomass on acid production of the mesophilic lactic acid bacteria strains tested

Strain (NCAIM)	**Average decrease in pH during fermentation compared to controls**							
	h 0	**h 2**	**h 4**	**h 6**	**h 8**	**h 10**	**h 12**	**h 14**
B.2125	-0.07	-0.07	-0.17	-0.16	-0.10	-0.02	0.00	0.00
B.2128	-0.05	-0.04	+0.03	+0.25*	+0.38*	+0.22*	+0.16*	+0.14*
B.2122	-0.05	-0.04	+0.02	+0.10	+0.26	+0.43	+0.50	+0.51
B.2123	-0.06	-0.06	-0.06	+0.01	+0.08*	+0.03	+0.03	+0.01
B.2126	-0.06	-0.07	-0.10*	-0.14	+0.01	+0.03	+0.03	+0.03*
B.2127	-0.04*	-0.03*	-0.02*	+0.22*	+0.54*	+0.61*	+0.51*	+0.58*
B.2124	-0.06*	-0.03	+0.04*	+0.14*	+0.30*	+0.19*	+0.18*	+0.14*
ATCC 19257	-0.05*	+0.03*	+0.12*	+0.56*	+0.59*	+0.14*	+0.04*	+0.06*
B.2120	-0.07	-0.04	0.00	+0.12*	+0.53*	+0.86*	+0.92*	+0.90*
B.1658	-0.05*	-0.05*	-0.07*	-0.03	+0.09	+0.10*	+0.09*	+0.10*

-: Retardation of acid production
+: Stimulation of acid production
* Significantly different at the *P* = 0.05 level (n = 6)

Used at the rate of 3 g/dm^3, Spirulina significantly increased ($P < 0.05$) the acid production of *Lc. lactis* subsp. *lactis* NCAIM B.2128, *Lc. lactis* subsp. *lactis* var. *diacetylactis* NCAIM B.2127, *Lc. lactis* subsp. *cremoris* ATCC 19257, *Lc. lactis* subsp. *cremoris* NCAIM B.2124 and *Leuconostoc mesenteroides* subsp. *cremoris* NCAIM B.2120 during the fermentation process.

Fig. 1 illustrates the changes in viable numbers of *Lc. lactis* subsp. *lactis* NCAIM B.2128, *Lc. lactis* subsp. *lactis* var. *diacetylactis* NCAIM B.2127 and *Lc. lactis* subsp. *cremoris* ATCC 19257 in cyanobacterial and control samples.

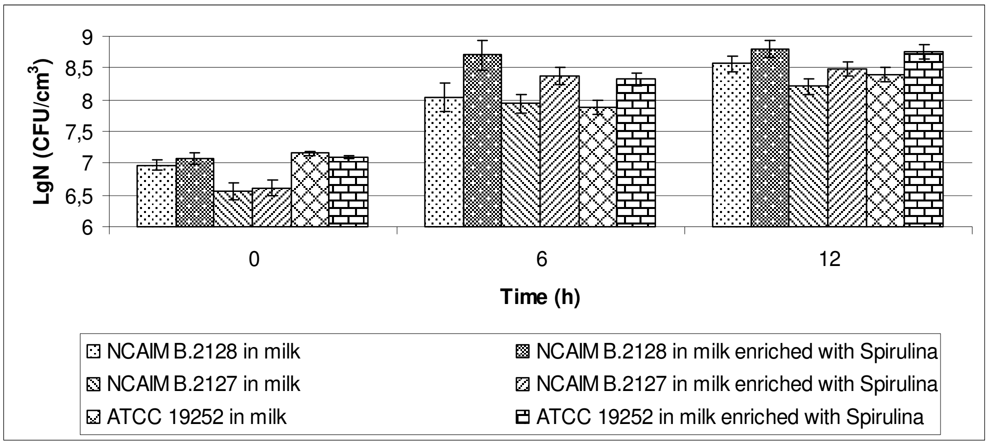

Fig. 1 Changes in viable cell counts of *Lactococcus lactis* subsp. *lactis* NCAIM B.2128, *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCAIM B.2127, and *Lactococcus lactis* subsp. *cremoris* ATCC 19257 during fermentation in milk and in Spirulina-enriched milk (whiskers indicate 95% confidence intervals of means) (n = 6)

By h 6 of the fermentation process, Spirulina enrichment resulted in a significant increase ($P < 0.05$) in the growth rates of all three strains tested. In the case of *Lc. lactis* subsp. *lactis* var. *diacetylactis* NCAIM B.2127 and *Lc.*

lactis subsp. *cremoris* ATCC 19257, significant differences ($P < 0.05$) were observed in viable counts at h 12 as well.

3.3. Antimicrobial properties of the Spirulina biomass

The 10 times diluted aqueous extract of Spirulina and the supernatant of its centrifuged homogenate had the highest inhibitory effect on *Sarcina* sp. of all the test organisms assayed. Other microorganisms whose growth was inhibited by Spirulina extracts included: *Acetobacter* sp., *Listeria monocytogenes* NCAIM B.01373, *Micrococcus luteus* T21, *Proteus mirabilis* HNCMB 61370, *Salmonella* Typhi-suis HNCMB 15016, *Staphylococcus aureus* HNCMB 112002, and *Staphylococcus epidermidis* HNCMB 110001. Aqueous extracts were superior to supernatants in terms of inhibitory properties. Ultrasonic cell disruption failed to improve the effectiveness of aqueous extracts.

3.4. Development of a functional fermented milk manufactured with mesophilic lactic acid bacteria and Spirulina biomass

According to the results of ranking tests done by sensory panelists, optimum organoleptic properties were achieved in the product formulation prepared with the mixed culture of *Lc. lactis* subsp. *lactis* NCAIM B.2128 and *Lc. lactis* subsp. *cremoris* ATCC 19257, and supplemented with sucrose at 10%, Spirulina biomass at 0.3%, and strawberry-kiwifruit flavor at 1.5%. **Table 2** illustrates the manufacturing technology of the novel functional fermented milk developed.

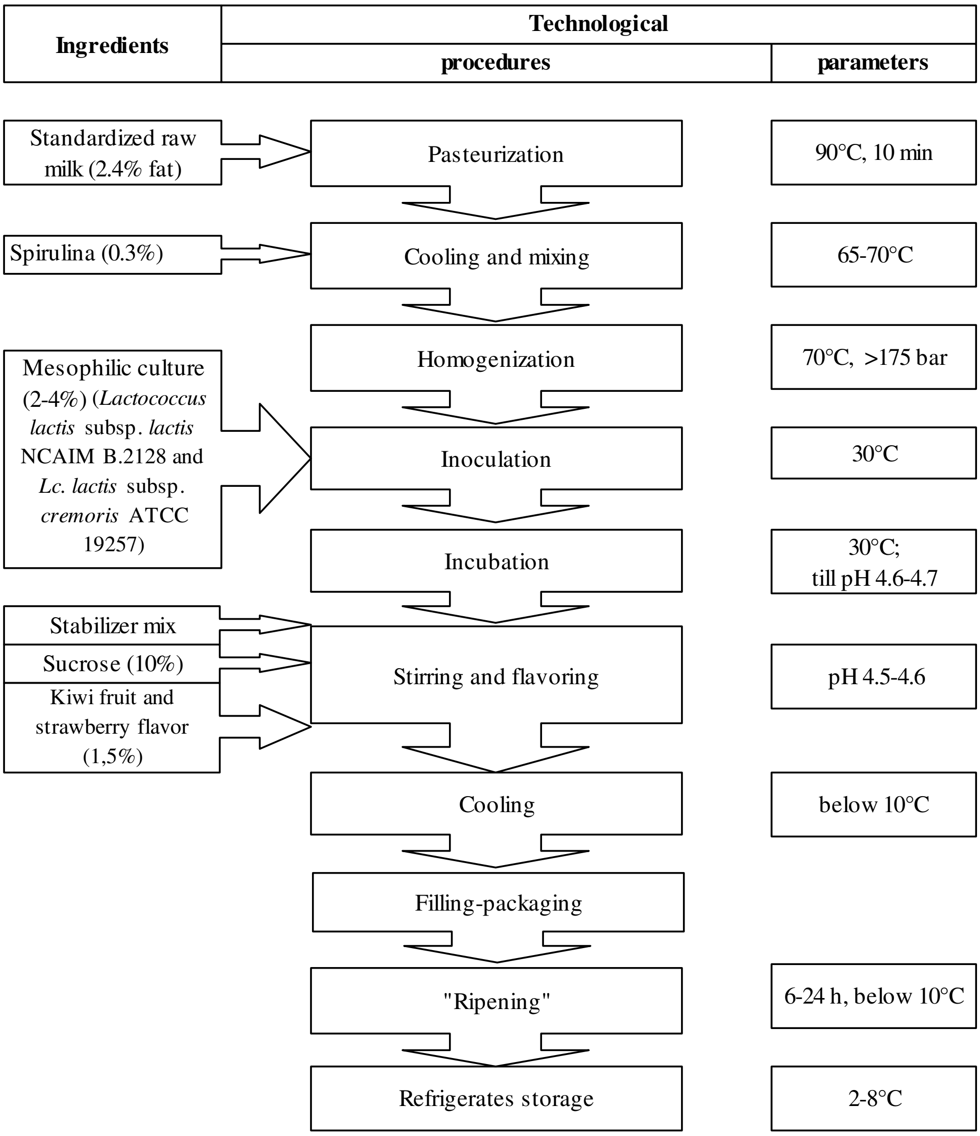

Fig. 2 Technology of manufacture for the novel Spirulina-enriched functional fermented milk

3.5. Influence of the Spirulina biomass on storage life of the newly developed product

During the first 2 weeks of refrigerated storage at 4°C, the *Lactococcus* counts were significantly higher ($P < 0.05$) in the cyanobacterial fermented milk than in the control product, confirming earlier reports on the stimulatory effects of Spirulina on coccus-shaped starter LAB. It is worth mentioning that an increase was observed in viable cell counts of both products during the first week of storage, however, viability percentages declined slowly thereafter. The lactococci counts of the two products did not differ significantly ($P > 0.05$) at the end of the 6-wk storage period.

Food regulations in Hungary require fermented milks to contain LAB of starter culture origin at concentrations of at least 10^7 CFU/g over the shelf life of the product. The lactococci counts in both products largely exceeded this value throughout the entire storage period.

4. NEW SCIENTIFIC FINDINGS

1. Used at the rate of 3 g/dm^3, Spirulina significantly increases ($P < 0.05$) the acid production by various strains of mesophilic lactic acid bacteria (*e.g.*, *Lactococcus lactis* subsp. *lactis* NCAIM B.2128, *Lc. lactis* subsp. *lactis* var. *diacetylactis* NCAIM B.2127, *Lc. lactis* subsp. *cremoris* ATCC 19257, *Lc. lactis* subsp. *cremoris* NCAIM B.2124, and *Leuconostoc mesenteroides* subsp. *cremoris* NCAIM B.2120) during fermentation in milk; and it also stimulates ($P < 0.05$) the growth of *Lc. lactis* subsp. *lactis* NCAIM B.2128, *Lc. lactis* subsp. *lactis* var. *diacetylactis* NCAIM B.2127, and *Lc. lactis* subsp. *cremoris* ATCC 19257.

2. Based on the results of agar diffusion assays, it is concluded that aqueous extracts from the Spirulina biomass are capable of inhibiting the growth of various foodborne pathogens and food spoilage microorganisms such as *Sarcina* sp., *Acetobacter* sp., *Listeria monocytogenes* NCAIM B.01373, *Micrococcus luteus* T21, *Proteus mirabilis* HNCMB 61370, *Salmonella* Typhi-suis HNCMB 15016, *Staphylococcus aureus* HNCMB 112002 and *Staphylococcus epidermidis* HNCMB 110001.

3. Patentable manufacturing technology for production of a novel Spirulina-enriched functional fermented milk has been developed. On the evidence of results from sensory evaluations, optimum organoleptic properties are achieved when the product is prepared with the mixed culture of *Lc. lactis* subsp. *lactis* NCAIM B.2128 and *Lc. lactis* subsp.

cremoris ATCC 19257, and is supplemented with sucrose at 10%, Spirulina biomass at 0.3%, and strawberry-kiwifruit flavor at 1.5%. During the first 2 weeks of refrigerated storage at 4±2°C, the Spirulina biomass significantly increases ($P < 0.05$) the viability of mesophilic starter bacteria in the product developed.

5. SCIENTIFIC PUBLICATIONS AND PRESENTATIONS ON THE TOPIC OF THE PH.D. DISSERTATION

Peer-Reviewed Papers

Gyenis, B., Szigeti, J., **Molnár, N.** & Varga, L. (2005) Use of dried microalgal biomasses to stimulate acid production and growth of *Lactobacillus plantarum* and *Enterococcus faecium* in milk. *Acta Agraria Kaposváriensis* **9** (2), 53–59.

Molnár, N., Gyenis, B. & Varga, L. (2005) Influence of a powdered *Spirulina platensis* biomass on acid production of lactococci in milk. *Milchwissenschaft* **60** (4), 380–382. **IF: 0.394**

Varga, L., **Molnár, N.** & Szigeti, J. (2005) The potential of *Spirulina* (*Arthrospira*) *platensis* to accumulate trace elements, and its dietary implications. *Acta Agronomica Óváriensis* **47** (1), 53–60.

Papers Published in Proceedings

Molnár, N., Szigeti, J. & Varga, L. (2004) Effect of a spray-dried *Arthrospira (Spirulina) platensis* biomass on acid development by various strains of *Lactococcus lactis* in milk. *Conference on Sustain Life – Secure Survival II "Socially and Environmentally Responsible Agribusiness"*. Proceedings, Prague, 5 pp.

Gyenis, B., Szigeti, J., **Molnár, N.** & Varga, L. (2004) *Lactobacillus plantarum* és *Enterococcus faecium* szaporodásának valamint savtermelésének serkentése tejben, szárított *Chlorella* és *Arthrospira* (*Spirulina*) biomassza felhasználásával (Use of dried *Chlorella* and *Arthrospira* (*Spirulina*) biomasses to stimulate growth and acid production of *Lactobacillus plantarum* and *Enterococcus faecium* in milk). *30th Day of Science in Óvár "Agricultural Production – In Harmony with Nature"*. Proceedings, Mosonmagyaróvár, 5 pp. (In Hungarian)

Abstracts

Varga, L., Szigeti, J. & **Molnár, N.** (2003) Trace element accumulation by *Spirulina platensis* (Cyanobacteria) and its dietary implications. *1st FEMS Congress of European Microbiologists*. Abstract Book, Ljubljana, 130.

Gyenis, B., Varga, L., Szigeti, J. & **Molnár, N.** (2004) Use of powdered microalgae to stimulate acid production and growth of *Lactobacillus plantarum* and *Enterococcus faecium* in milk. *American Dairy Science Association – American Society of Animal Science – Poultry Science Association 2004 Joint Annual Meeting*. Abstracts, St. Louis, Missouri: *Journal of Animal Science* **82** (Supplement 1) / *Journal of Dairy Science* **87** (Supplement 1) / *Poultry Science* **83** (Supplement 1) 165. **IF: 2.134**

Molnár, N., Varga, L., Szigeti, J. & Gyenis, B. (2004) Influence of an *Arthrospira* (*Spirulina*) *platensis* biomass on acid production of lactococci. *American Dairy Science Association – American Society of Animal Science – Poultry Science Association 2004 Joint Annual Meeting*. Abstracts, St. Louis, Missouri: *Journal of Animal Science* **82** (Supplement 1) / *Journal of Dairy Science* **87** (Supplement 1) / *Poultry Science* **83** (Supplement 1) 382–383. **IF: 2.134**

Varga, L., Gyenis, B., **Molnár, N.** & Szigeti, J. (2004) Effect of inulin on the microflora of an ABT-type fermented milk during refrigerated storage. *American Dairy Science Association – American Society of Animal Science – Poultry Science Association 2004 Joint Annual Meeting*. Abstracts, St. Louis, Missouri: *Journal of Animal Science* **82** (Supplement 1) / *Journal of Dairy Science* **87** (Supplement 1) / *Poultry Science* **83** (Supplement 1) 164. **IF: 2.134**