Acta Agraria Kaposváriensis (2005) Vol 9 No 2, 53-59
Kaposvári Egyetem, Állattudományi Kar, Kaposvár
University of Kaposvár, Faculty of Animal Science, Kaposvár

Use of dried microalgal biomasses to stimulate acid production and growth of *Lactobacillus plantarum* and *Enterococcus faecium* in milk

B. Gyenis, J. Szigeti, N. Molnár, L. Varga
Institute of Food Science, Faculty of Agricultural and Food Sciences, University of West Hungary
Mosonmagyaróvár, H-9200 Lucsony u. 15-17.

ABSTRACT

Microalgae have been commercially cultured for nearly four decades with the main species grown being Chlorella *and* Spirulina*. The effect of dried* Spirulina platensis *and* Chlorella vulgaris *biomasses, added at a concentration of 3 g/dm^3, on acid production and growth of* Lactobacillus plantarum *and* Enterococcus faecium *strains used for feed fermentation purposes was evaluated in milks with total solids contents ranging from 12% to 30%. Our results showed that acid development by and growth rate of* L. plantarum *and* E. faecium *were stimulated significantly (P<0.05) by* S. platensis *and* C. vulgaris*, respectively, in all culture media formulations tested. In conclusion, the powdered* Chlorella *and* Spirulina *biomasses rich in biologically active compounds are potentially suitable for use in cost-effective production of novel, milk-based fermented feeds.*
(Keywords: *Chlorella vulgaris*, *Spirulina platensis*, *Enterococcus faecium*, *Lactobacillus plantarum*, milk)

ÖSSZEFOGLALÁS

Lactobacillus plantarum és *Enterococcus faecium* savtermelésének valamint szaporodásának serkentése tejben, szárított mikroalga biomasszák felhasználásával

Gyenis B., Szigeti J., Molnár N., Varga L.
Nyugat-magyarországi Egyetem, Mezőgazdaság- és Élelmiszer-tudományi Kar, Élelmiszer-tudományi Intézet
Mosonmagyaróvár, 9200 Lucsony u. 15-17.

A mikroalgák (elsősorban a Chlorella *és* Spirulina *fajok) kereskedelmi célú termesztése közel négy évtizedes múltra tekint vissza. Kísérleteink során szárított, 3 g/dm^3 koncentrációban alkalmazott* Spirulina platensis, *ill.* Chlorella vulgaris *biomasszának takarmányfermentálásra használt* Lactobacillus plantarum *és* Enterococcus faecium *törzsek szaporodására és savtermelésére gyakorolt hatását teszteltük 12-30% szárazanyag-tartalmú modell tej-tápközegekben. A kapott eredmények azt mutatták, hogy a* S. platensis, *ill. a* C. vulgaris *szárított biomasszájának adagolása szignifikánsan (P<0,05) serkentette a* L. plantarum *és az* E. faecium *szaporodási sebességét, továbbá savtermelő aktivitását, az összes alkalmazott tápközegben. Megállapítható, hogy a bioaktív komponensekben gazdag, szárított* Chlorella *ill.* Spirulina *biomassza potenciálisan alkalmas új típusú, tejalapú fermentált takarmányok gazdaságos előállítására.*
(Kulcsszavak: *Chlorella vulgaris*, *Spirulina platensis*, *Enterococcus faecium*, *Lactobacillus plantarum*, tej)

INTRODUCTION

Microalgae are photosynthetic microorganisms that can be used to produce high value compounds (*Kreitlow et al.*, 1999). Spray-dried microalgal biomasses typically contain 3% to 7% moisture, 46% to 63% protein, 8% to 17% carbohydrates, 4% to 22% lipids, 2% to 4% nucleic acid, 7% to 10% ash, and a wide range of vitamins and other biologically active substances. Microalgae have been commercially produced for approximately 40 years now with the main species grown being *Chlorella* and *Spirulina* for health food (*Borowitzka*, 1999). *Chlorella vulgaris* is a green algal species that produces astaxanthin, canthaxanthin and, in minor amounts, β-carotene and luthein (*Mendes et al.*, 2003). *Spirulina platensis* is a planktonic cyanobacterium belonging to prokaryotic algae. It produces γ-linolenic acid in large amounts (*Cohen*, 1997).

Particular microorganisms such as *Lactobacillus plantarum* or *Enterococcus faecium* have been increasingly used as probiotics in animal nutrition for more than 15 years, and have been strictly regulated since 1993 (*Vescovo et al.*, 1993; *McAllister et al.*, 1998; *Becquet*, 2003). *Lactobacillus plantarum* is a Gram-positive, non-motile, non-sporeforming bacterium. Its cells are straight rods with rounded ends, occurring singly, in pairs or in short chains. *Lactobacillus plantarum* is a widely distributed species in most fermented products of animal and plant origin, where it is either used in controlled fermentation or is derived from the environment (*Corsetti & Gobbetti*, 2003). As for *E. faecium*, it is a Gram-positive, catalase-negative, coccus-shaped bacterium, characterized by its capability to grow at 10°C and 45°C, in 6.5% NaCl at pH 9.6 and its ability to survive heating at 60°C for 30 min. Thus, it is among the most thermotolerant species of non-sporeforming bacteria. *Enterococcus faecium* is significant in dairy manufacturing by having both beneficial and detrimental effects in products. Beneficial effects include desirable flavor enhancement, bacteriocin production, and probiotic impact, whereas detrimental effects include product spoilage (*Flint*, 2003).

Varga et al. (1999) reported that a cyanobacterial biomass significantly stimulated ($P<0.05$) growth and acid production of thermophilic dairy starter bacteria, therefore, it proved to be suitable for cost-effective manufacture of novel functional fermented dairy foods. The aim of this work was to test the capability of *Spirulina* and *Chlorella* microalgal biomasses, in milks with various total solids contents, to stimulate selected lactobacilli and enterococci used for feed fermentation purposes.

MATERIALS AND METHODS

Reconstituted skim milks with total solids contents ranging from 12% to 30% were used as raw material, which were heated to 90°C and held for 10 min before being cooled to inoculation temperature.

The *L. plantarum* and *E. faecium* freeze-dried starter cultures were kindly supplied by the Department of Animal Nutrition, University of West Hungary (Mosonmagyaróvár, Hungary). Before the start of the trials, the strains were subcultured twice at 30°C for 24 h in De Man–Rogosa–Sharpe (MRS) broth and MRS agar (*L. plantarum*) and at 37°C for 24 h in Casein-peptone Soymeal-peptone (CASO) broth and Citrate Azide Tween® Carbonate (CATC) agar (*E. faecium*). All these culture media were purchased from Merck (Darmstadt, Germany).

The *S. platensis* and *C. vulgaris* biomasses were obtained from the Institute of Cereal Processing (Bergholz-Rehbrücke, Germany). Previous work (*Springer et al.*,

1998) indicated that 3 g/dm^3 of microalgal biomass was optimal in regards to sensory properties and cost.

The heat-treated and cooled microalgae-supplemented and control milks were inoculated with *L. plantarum* or *E. faecium* at the rate of 1%, corresponding to approximately 6.5×10^6 cfu/cm^3 of milk, and were then incubated at 30°C or 37°C, respectively.

The pH value of three replicate samples from each treatment at each sampling time was measured with an HI 8521 pH-meter and combined glass electrode (Hanna Instruments, Karlsruhe, Germany).

Viable cell counts were determined by using the standard pour-plate technique. MRS agar was employed for enumeration of *L. plantarum*. The plates were incubated at 30°C for 24 to 48 h. CATC agar was used to enumerate *E. faecium*. The inoculated plates were incubated at 37°C for 24 to 48 h. The entire experimental program was repeated twice.

The influence of microalgal biomasses on acid production and growth of *L. plantarum* and *E. faecium* during the fermentation process was analyzed with the Student's *t*-test, by means of the STATISTICA data analysis software system, version 6.1 (StatSoft, Tulsa, OK, USA). Significance of difference was set at $P<0.05$ in all cases.

RESULTS AND DISCUSSION

Tables 1 to 3 show the results obtained.

Table 1

Effect of 3 g/dm^3 *Chlorella vulgaris* biomass on acid production[1] of *Enterococcus faecium* in milks with total solids contents ranging between 12% and 30%

Time h (1)	Milk with							
	12% total solids (2)		18% total solids (3)		24% total solids (4)		30% total solids (5)	
	Control (6)	*Chlorella* (7)	Control (6)	*Chlorella* (7)	Control (6)	*Chlorella* (7)	Control (6)	*Chlorella* (7)
0	6.31±0.07^a	6.31±0.08^a	6.33±0.06^a	6.33±0.05^a	6.34±0.07^a	6.34±0.09^a	6.31±0.08^a	6.31±0.06^a
10	6.13±0.08^a	5.64±0.12^b	6.06±0.09^a	5.20±0.07^b	5.95±0.06^a	5.15±0.12^b	5.84±0.06^a	5.23±0.08^b
12	5.76±0.07^a	5.37±0.10^b	5.80±0.08^a	4.92±0.09^b	5.83±0.09^a	4.91±0.11^b	5.64±0.07^a	4.89±0.06^b
14	5.40±0.09^a	5.04±0.08^b	5.42±0.08^a	4.55±0.11^b	5.70±0.08^a	4.50±0.08^b	5.43±0.09^a	4.55±0.06^b
17	5.32±0.06^a	4.44±0.08^b	5.36±0.06^a	4.48±0.05^b	5.41±0.07^a	4.19±0.07^b	5.39±0.09^a	4.52±0.06^b
20	5.13±0.07^a	4.10±0.07^b	5.14±0.08^a	4.16±0.07^b	5.14±0.06^a	4.07±0.09^b	5.21±0.10^a	4.51±0.09^b
22	5.07±0.05^a	4.04±0.06^b	5.08±0.05^a	4.06±0.10^b	5.06±0.08^a	3.92±0.06^b	5.15±0.06^a	4.49±0.10^b

[1]Values are pH means±SD based on 6 observations: 3 samples, 2 replicates. ([1]*Az adatok 6 mérés – 3 párhuzamos×2 ismétlés – pH-átlagát±szórását jelölik.*); [a,b]Values bearing different superscript letters within a row in the same total solids subcolumns differ significantly. ([a,b]*Az azonos szárazanyag-tartalmat jelző oszlopok ugyanazon soraiban szereplő eltérő betűk szignifikáns különbséget jeleznek.*) ($P<0.05$)

1. táblázat: 3 g/dm^3 Chlorella vulgaris *biomassza hatása* Enterococcus faecium *savtermelésére[1] 12-30% szárazanyag-tartalmú tej-tápközegekben*

Idő, óra(1), 12% szárazanyag-tartalmú tej(2), 18% szárazanyag-tartalmú tej(3), 24% szárazanyag-tartalmú tej(4), 30% szárazanyag-tartalmú tej(5), Kontroll(6), Chlorellá*val kiegészített(7)*

Table 2

Effect of 3 g/dm^3 *Spirulina platensis* biomass on acid production[1] of *Lactobacillus plantarum* in milks with total solids contents ranging between 12% and 30%

Time h (1)	Milk with							
	12% total solids (2)		18% total solids (3)		24% total solids (4)		30% total solids (5)	
	Control (6)	*Spirulina* (7)	Control (6)	*Spirulina* (7)	Control (6)	*Spirulina* (7)	Control (6)	*Spirulina* (7)
0	6.45±0.06^a	6.48±0.05^a	6.47±0.06^a	6.47±0.06^a	6.45±0.08^a	6.47±0.07^a	6.47±0.06^a	6.47±0.05^a
10	5.92±0.05^a	5.37±0.07^b	5.93±0.06^a	5.54±0.09^b	5.94±0.07^a	5.55±0.11^b	5.95±0.06^a	5.74±0.05^b
12	5.73±0.06^a	5.09±0.09^b	5.76±0.07^a	5.32±0.10^b	5.75±0.06^a	5.31±0.10^b	5.83±0.05^a	5.55±0.09^b
14	5.55±0.08^a	4.95±0.06^b	5.60±0.08^a	5.17±0.11^b	5.61±0.09^a	5.16±0.12^b	5.70±0.08^a	5.41±0.06^b
17	5.40±0.10^a	4.81±0.05^b	5.38±0.06^a	5.00±0.07^b	5.47±0.06^a	5.02±0.12^b	5.50±0.05^a	5.23±0.06^b
20	5.24±0.11^a	4.71±0.08^b	5.29±0.05^a	4.93±0.08^b	5.34±0.09^a	4.94±0.08^b	5.42±0.07^a	5.15±0.05^b
22	5.15±0.06^a	4.62±0.10^b	5.20±0.07^a	4.85±0.06^b	5.29±0.08^a	4.86±0.09^b	5.34±0.05^a	5.10±0.06^b

[1, a, b]See Table 1 (*lásd 1. táblázat*)

2. táblázat: 3 g/dm^3 Spirulina platensis *biomassza hatása* Lactobacillus plantarum *savtermelésére[1] 12-30% szárazanyag-tartalmú tej-tápközegekben*

(1-6) Lásd 1. táblázat, Spirulinával *kiegészített(7)*

Table 3

Effect of 3 g/dm^3 *Chlorella vulgaris* biomass on growth[1] of *Lactobacillus plantarum* and *Enterococcus faecium* in milk with 12% total solids content

Time, h (1)	Control (2)	*Chlorella*-enriched (3)	Control (4)	*Chlorella*-enriched (5)
	milk inoculated with			
	Lactobacillus plantarum		*Enterococcus faecium*	
0	6.78±0.12^a	6.88±0.08^a	6.83±0.10^a	6.93±0.09^a
8	8.18±0.09^b	8.52±0.07^a	8.26±0.09^b	8.66±0.08^a
12	8.31±0.10^b	8.92±0.09^a	8.41±0.11^b	8.96±0.06^a
22	8.61±0.10^b	8.98±0.08^a	8.72±0.10^b	9.08±0.07^a

[1]Values are log cfu/cm^3 means±SD, based on 6 observations: 3 samples, 2 replicates. (*[1]Az adatok 6 vizsgálat – 3 párhuzamos×2 ismétlés – log cfu/cm^3-átlagát±szórását jelölik.*); [a,b]Values bearing different superscript letters within a row in the same bacterial subcolumns differ significantly. (*[a,b]Az azonos baktériumfajt jelző oszlopok ugyanazon soraiban szereplő eltérő betűk szignifikáns különbséget jeleznek.*) (P<0.05)

3. táblázat: 3 g/dm^3 Chlorella vulgaris *biomassza hatása* Lactobacillus plantarum *és* Enterococcus faecium *szaporodására[1] 12% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Lactobacillus plantarum*mal beoltott kontroll-tej(2),* Lactobacillus plantarum*mal beoltott* Chlorella-*tartalmú tej(3),* Enterococcusus faecium*mal beoltott kontroll-tej(4),* Enterococcus faecium*mal beoltott* Chlorella-*tartalmú tej(5)*

As can be seen, acid production and growth of *E. faecium* and *L. plantarum* were stimulated significantly ($P<0.05$) by *C. vulgaris* and *S. platensis*, respectively, in all culture media formulations used. Our findings are consistent with those of *Varga et al.* (1999), who demonstrated that acid production and growth rate of thermophilic dairy starter cultures, such as *Streptococcus thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium bifidum*, could be stimulated significantly ($P<0.05$) by a *S. platensis* biomass. In accordance with previous reports by various authors (*Shirota et al.*, 1964; *Stengel*, 1970; *Zielke et al.*, 1978; *Kurita et al.*, 1979; *Webb*, 1982), the substances responsible for the stimulatory properties of this cyanobacterial biomass were identified as adenine, hypoxanthine, and free amino acids (*Varga et al.*, 1999).

Considerable work on acid production of *Enterococcus* species in milk has been reported. In general, enterococci exhibit low milk acidifying ability (*Giraffa*, 2003). Recent investigations on enterococci of dairy origin confirmed the poor acidifying capacity of these microorganisms in milk with only a small percentage of the strains showing a pH below 5.0 to 5.2 after 16 to 24 h of incubation at 37°C (*Andrighetto et al.*, 2001; *Durlu-Ozkaya et al.*, 2001; *Sarantinopoulos et al.*, 2001). It was also demonstrated that *E. faecalis* is generally a stronger acidifier than *E. faecium*. A high acidifying potential in skim milk with a pH lowering to approximately 4.5 after 24 h of fermentation was observed for *E. faecalis* strains isolated from an Italian artisanal cheese (*Giraffa et al.*, 1993; *Suzzi et al.*, 2000). The specific enterococcal strain used in our trial showed good acidification properties by lowering the pH of control milks to between 5.06 and 5.15 after 22 h of fermentation at 37°C (*Table 1*). The acidity levels of 3.92 to 4.49 reached by the same *E. faecium* strain in *Chlorella*-supplemented milks under identical conditions were even lower than the value of 4.5 reported by *Giraffa et al.* (1993) and *Suzzi et al.* (2000) for the strong acidifier *E. faecalis*.

Lactobacillus plantarum proved to be a slightly poorer acidifier than *E. faecium* because the pH value of products ranged from 5.15 to 5.34 and from 4.62 to 5.10 in control and *Spirulina*-enriched samples, respectively, after 22 h of fermentation at 30°C. However, similar to what was experienced with *E. faecium*, the addition of microalgal biomass had a significant stimulatory effect ($P<0.05$) on *L. plantarum* throughout the entire fermentation process (*Table 2*).

CONCLUSIONS

The stimulatory properties of microalgal biomasses on acid production and growth of *L. plantarum* and *E. faecium* are of practical importance because, thus, shorter time is needed for the manufacture of the same amount of fermented feed and, consequently, productivity will improve. In addition, a rapid rate of acid production also prevents the growth of undesirable microorganisms. Therefore, *Chlorella* and *Spirulina* biomasses rich in bioactive compounds are potentially suitable for use in cost-effective production of novel, milk-based fermented feeds.

ACKNOWLEDGMENT

Author L. Varga is grateful to the Hungarian Academy of Sciences for the award of a János Bolyai Research Scholarship.

REFERENCES

Andrighetto, C., Knijff, E., Lombardi, A., Torriani, S., Vancanneyt, M., Kersters, K., Swings, J., Dellaglio, F. (2001). Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. J. Dairy Res., 68. 303–316.

Becquet, P. (2003). EU assessment of enterococci as feed additives. Int. J. Food Microbiol., 88. 247–254.

Borowitzka, M.A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters. J. Biotechnol., 70. 313–321.

Cohen, Z. (1997). The chemicals of *Spirulina*. In: Vonshak, A. (ed.) *Spirulina platensis* (*Arthrospira*): Physiology, Cell-biology and Biotechnology. Taylor and Francis Ltd., London. 175–204.

Corsetti, A., Gobbetti, M. (2003). *Lactobacillus plantarum*. In: Roginski, H., Fuquay, J.W., Fox, P.F. (eds) Encyclopedia of Dairy Sciences. Vol. 3. Academic Press & Elsevier Science, Amsterdam, Boston, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo. 1501–1507.

Durlu-Ozkaya, F., Xanthopoulos, V., Tunail, N., Litopoulou-Tzanetaki, E. (2001). Technologically important properties of lactic acid bacteria isolates from Beyaz cheese made from raw ewes' milk. J. Appl. Microbiol., 91. 861–870.

Flint, S. (2003). *Enterococcus faecalis* and *Enterococcus faecium*. In: Roginski, H., Fuquay, J.W., Fox, P.F. (eds) Encyclopedia of Dairy Sciences. Vol. 2. Academic Press & Elsevier Science, Amsterdam, Boston, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo. 904–907.

Giraffa, G. (2003). Functionality of enterococci in dairy products. Int. J. Food Microbiol., 88. 215–222.

Giraffa, G., Gatti, M., Carminati, D., Neviani, E. (1993). Biochemical and metabolic characteristics of strains belonging to *Enterococcus* genus isolated from dairy products. Proceedings of the Congress on Biotechnology and Molecular Biology of Lactic Acid Bacteria for the Improvement of Foods and Feeds Quality, Naples, February 23-24.

Kreitlow, S., Mundt, S., Lindequist, U. (1999). Cyanobacteria — a potential source of new biologically active substances. J. Biotechnol., 70. 61–63.

Kurita, H., Tajima, O., Fukimbara, T. (1979). Isolation and identification of nucleosides in *Chlorella* extract. J. Agr. Chem. Soc. Japan, 53(4). 131–133.

McAllister, T.A., Feniuk, R., Mir, Z., Mir, P., Selinger, L.B., Cheng, K.J. (1998). Inoculants for alfalfa silage: effects on aerobic stability, digestibility and the growth performance of feedlot steers. Livestock Prod. Sci., 53. 171–181.

Mendes, R.L., Nobre, B.P., Cardoso, M.T., Pereira, A.P., Palavra, A.F. (2003). Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. Inorg. Chim. Acta, 356. 328–334.

Sarantinopoulos, P., Andrighetto, C., Georgalaki, M.D., Rea, M.C., Lombardi, A., Cogan, T.M., Kalantzopoulos, G., Tsakalidou, E. (2001). Biochemical properties of enterococci relevant to their technological performance. Int. Dairy J., 11. 621–647.

Shirota, M., Nagamatsu, N., Takechi, Y. (1964). Method for cultivating lactobacilli. U.S. Pat. No. 3,123,538.

Springer, M., Pulz, O., Szigeti, J., Ördög, V., Varga, L. (1998). Verfahren zur Herstellung von biologisch hochwertigen Sauermilcherzeugnissen. Eur. Pat. No. DE 19,654,614,A1.

Stengel, E. (1970). Anlagentypen und Verfahren der technischen Algenmassenproduktion. Ber. Dtsch. Bot. Ges., 83. 589–606.

Suzzi, G., Lombardi, A., Lanorte, M.T., Caruso, M., Andrighetto, C., Gardini, F. (2000). Characterization of autochthonous enterococci isolated from Semicotto Caprino cheese, a traditional cheese produced in Southern Italy. J. Appl. Microbiol., 89. 267–274.

Varga, L., Szigeti, J., Ördög, V. (1999). Effect of a *Spirulina platensis* biomass and that of its active components on single strains of dairy starter cultures. Milchwissenschaft, 54. 187–190.

Vescovo, M., Torriani, S., Dellaglio, F., Botazzi, V. (1993). Basic characteristics, ecology and applications of *Lactobacillus plantarum*: a review. Annali di Microbiologia ed Enzimologia, 43. 261–284.

Webb, L.E. (1982). Detection by Warburg manometry of compounds stimulatory to lactic acid bacteria. J. Dairy Res., 49. 479–486.

Zielke, H., Kneifel, H., Webb, L.E., Soeder, C.J. (1978). Stimulation of lactobacilli by an aqueous extract of the green alga *Scenedesmus acutus* 276−3a. Eur. J. Appl. Microbiol. Biotechnol., 6. 79–86.

Corresponding author (*levelezési cím*):

László Varga
Department of Dairy Science, Institute of Food Science, Faculty of Agricultural and Food Sciences, University of West Hungary
H-9200 Mosonmagyaróvár, Lucsony u. 15-17.
Nyugat-magyarországi Egyetem, Mezőgazdaság- és Élelmiszer-tudományi Kar, Élelmiszer-tudományi Intézet, Tejgazdaságtani Tanszék
9200 Mosonmagyaróvár, Lucsony u. 15-17.
Tel.: +36 96 566 652, fax: +36 96 566 653
e-mail: VargaL@mtk.nyme.hu