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

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ABSTRACT

Microalgae are photosynthetic microorganisms that can be used to produce a wide range of high value compounds. They have been commercially cultured for nearly four decades with the main species grown being Chlorella *and* Spirulina*. The effect of spray-dried* Spirulina platensis *and* Chlorella vulgaris *biomasses, added at a concentration of 3 g/L, on acid production and growth of* Lactobacillus plantarum *and* Enterococcus faecium *strains primarily used for feed fermentation purposes was evaluated in milks with total solids contents ranging from 12% to 30%. The acid development by and growth rate of* L. plantarum *and* E. faecium *were found to be stimulated significantly ($P < 0.05$) by* S. platensis *and* C. vulgaris*, respectively in all culture media formulations used. The dry matter content of milks did not influence the growth and acidification properties of the starter organisms tested. In conclusion, the powdered* Chlorella *and* Spirulina *biomasses rich in biologically active compounds are potentially suitable for use in cost-effective production of milk-based functional 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

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A mikroalgák fotoszintetizáló szervezetek, amelyek számos értékes vegyület előállítására képesek. A Chlorella *és a* Spirulina *mikroalga-fajok kereskedelmi célú termesztése közel négy évtizedes múltra tekint vissza. Kísérleteink során porlasztva szárított, 3 g/L 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 *és a* C. vulgaris *szárított biomasszájának adagolása szignifikáns mértékben ($P < 0,05$) serkentette a* L. plantarum *és az* E. faecium *szaporodási sebességét és savtermelő aktivitását, az összes alkalmazott tápközegben; a tej-tápközegek szárazanyag-tartalma viszont nem befolyásolta a vizsgált startertörzsek szaporodási és savtermelési mutatóit. Összefoglalásképpen megállapítható, hogy a bioaktív komponensekben gazdag, szárított* Chlorella *és* Spirulina *biomassza alkalmas funkcionális hatású, fermentált, tejalapú 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, 8% to 10% fiber, 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 and emerges after manufacturing (*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 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 milk powders 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 MRS broth and MRS agar (*L. plantarum*) and at 37°C for 24 h in TSB broth and SB agar (*E. faecium*). All these culture media were purchased from Merck KGaA (Darmstadt, Germany).

The *S. platensis* and *C. vulgaris* biomasses were obtained from the Institute of Cereal Processing, Inc. (Bergholz-Rehbrücke, Germany). Previous work (*Springer et al.*, 1998) indicated that 3 g/L 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% 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 Deutschland GmbH, Karlsruhe, Germany) standardized with pH 4.01 and 7.01 standard buffer solutions. Viable cell counts were also determined using the standard pour-plate technique. 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 Inc., Tulsa, OK). Significance of difference was set at $P < 0.05$ in all cases.

RESULTS AND DISCUSSION

Tables 1 to 10 show the results obtained.

Table 1
Table 2
Table 3
Table 4
Table 5
Table 6
Table 7
Table 8
Table 9
Table 10

It is clearly visible that acid production and growth of *L. plantarum* and *E. faecium* were found to be stimulated significantly ($P < 0.05$) by *S. platensis* and *C. vulgaris*, respectively, in all culture media formulations used. These findings are in accordance with previous reports by *Varga et al.* (1999), who stated 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. 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 seen 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. 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*-supplemented 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.

It is also worth noting that the dry matter content of milks did not influence the growth and acidification properties of the starter organisms tested in this study.

In conclusion, the *Chlorella* and *Spirulina* biomasses rich in bioactive compounds are potentially suitable for use in cost-effective production of milk-based functional fermented feeds.

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Table 1

Effect of 3 g/L *Spirulina platensis* biomass on acid production[1] of *Lactobacillus plantarum* in milk with 30% total solids content

Time, h (1)	Control (2)	*Spirulina*-supplemented (3)
0	6.47[a]	6.47[a]
10	5.95[a]	5.74[b]
12	5.83[a]	5.55[b]
14	5.70[a]	5.41[b]
17	5.50[a]	5.23[b]
20	5.42[a]	5.15[b]
22	5.34[a]	5.10[b]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).
[1]Figures are mean pH values, based on six observations: three samples, two replicates ([1]Az adatok 6 mérés – 3 párhuzamos x 2 ismétlés – pH-átlagát jelölik).

1. táblázat: 3 g/L Spirulina platensis *biomassza hatása* Lactobacillus plantarum *savtermelésére[1] 30% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Kontroll(2), Spirulin*ával kiegészített(3)*

Table 2

Effect of 3 g/L *Spirulina platensis* biomass on acid production[1] of *Lactobacillus plantarum* in milk with 24% total solids content

Time, h (1)	**Control (2)**	***Spirulina*-supplemented (3)**
0	6.35[b]	6.47[a]
10	5.94[a]	5.55[b]
12	5.75[a]	5.31[b]
14	5.61[a]	5.16[b]
17	5.47[a]	5.02[b]
20	5.34[a]	4.94[b]
22	5.29[a]	4.86[b]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).

[1]Figures are mean pH values, based on six observations: three samples, two replicates ([1]Az adatok 6 mérés – 3 párhuzamos x 2 ismétlés – pH-átlagát jelölik).

2. táblázat: 3 g/L Spirulina platensis *biomassza hatása* Lactobacillus plantarum *savtermelésére*[1] *24% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Kontroll(2), Spirulin*ával kiegészített(3)*

Table 3

Effect of 3 g/L *Spirulina platensis* biomass on acid production[1] of *Lactobacillus plantarum* in milk with 18% total solids content

Time, h (1)	**Control (2)**	***Spirulina*-supplemented (3)**
0	6.47[a]	6.47[a]
10	5.93[a]	5.54[b]
12	5.76[a]	5.32[b]
14	5.60[a]	5.17[b]
17	5.38[a]	5.00[b]
20	5.29[a]	4.93[b]
22	5.20[a]	4.85[b]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).
[1]Figures are mean pH values, based on six observations: three samples, two replicates ([1]Az adatok 6 mérés – 3 párhuzamos x 2 ismétlés – pH-átlagát jelölik).

3. táblázat: 3 g/L Spirulina platensis *biomassza hatása* Lactobacillus plantarum *savtermelésére[1] 18% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Kontroll(2), Spirulin*ával kiegészített(3)*

Table 4

Effect of 3 g/L *Spirulina platensis* biomass on acid production[1] of *Lactobacillus plantarum* in milk with 12% total solids content

Time, h (1)	**Control (2)**	***Spirulina*-supplemented (3)**
0	6.35[b]	6.48[a]
10	5.92[a]	5.37[b]
12	5.73[a]	5.09[b]
14	5.55[a]	4.95[b]
17	5.40[a]	4.81[b]
20	5.24[a]	4.71[b]
22	5.15[a]	4.62[b]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).
[1]Figures are mean pH values, based on six observations: three samples, two replicates ([1]Az adatok 6 mérés – 3 párhuzamos x 2 ismétlés – pH-átlagát jelölik).

4. táblázat: 3 g/L Spirulina platensis *biomassza hatása* Lactobacillus plantarum *savtermelésére[1] 12% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Kontroll(2), Spirulin*ával kiegészített(3)*

Table 5

Effect of 3 g/L *Chlorella vulgaris* biomass on acid production[1] of *Enterococcus faecium* in milk with 30% total solids content

Time, h (1)	**Control (2)**	***Chlorella*-supplemented (3)**
0	6.31[a]	6.31[a]
10	5.84[a]	5.23[b]
12	5.64[a]	4.89[b]
14	5.43[a]	4.55[b]
17	5.39[a]	4.52[b]
20	5.21[a]	4.51[b]
22	5.15[a]	4.49[b]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).
[1]Figures are mean pH values, based on six observations: three samples, two replicates ([1]Az adatok 6 mérés – 3 párhuzamos x 2 ismétlés – pH-átlagát jelölik).

5. táblázat: 3 g/L Chlorella vulgaris *biomassza hatása* Enterococcus faecium *savtermelésére[1] 30% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Kontroll(2), Chlorell*ával kiegészített(3)*

Table 6

Effect of 3 g/L *Chlorella vulgaris* biomass on acid production[1] of *Enterococcus faecium* in milk with 24% total solids content

Time, h (1)	Control (2)	*Chlorella*-supplemented (3)
0	6.34[a]	6.34[a]
10	5.95[a]	5.15[b]
12	5.83[a]	4.91[b]
14	5.70[a]	4.50[b]
17	5.41[a]	4.19[b]
20	5.14[a]	4.07[b]
22	5.06[a]	3.92[b]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).
[1]Figures are mean pH values, based on six observations: three samples, two replicates ([1]Az adatok 6 mérés – 3 párhuzamos x 2 ismétlés – pH-átlagát jelölik).

6. táblázat: 3 g/L Chlorella vulgaris *biomassza hatása* Enterococcus faecium *savtermelésére[1] 24% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Kontroll(2), Chlorell*ával kiegészített(3)*

Table 7

Effect of 3 g/L *Chlorella vulgaris* biomass on acid production[1] of *Enterococcus faecium* in milk with 18% total solids content

Time, h (1)	**Control (2)**	***Chlorella*-supplemented (3)**
0	6.33[a]	6.33[a]
10	6.06[a]	5.20[b]
12	5.80[a]	4.92[b]
14	5.42[a]	4.55[b]
17	5.36[a]	4.48[b]
20	5.14[a]	4.16[b]
22	5.08[a]	4.06[b]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).
[1]Figures are mean pH values, based on six observations: three samples, two replicates ([1]Az adatok 6 mérés – 3 párhuzamos x 2 ismétlés – pH-átlagát jelölik).

7. táblázat: 3 g/L Chlorella vulgaris *biomassza hatása* Enterococcus faecium *savtermelésére[1] 18% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Kontroll(2), Chlorell*ával kiegészített(3)*

Table 8

Effect of 3 g/L *Chlorella vulgaris* biomass on acid production[1] of *Enterococcus faecium* in milk with 12% total solids content

Time, h (1)	**Control (2)**	***Chlorella*-supplemented (3)**
0	6.31[a]	6.31[a]
10	6.13[a]	5.64[b]
12	5.76[a]	5.37[b]
14	5.40[a]	5.04[b]
17	5.32[a]	4.44[b]
20	5.13[a]	4.10[b]
22	5.07[a]	4.04[b]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).
[1]Figures are mean pH values, based on six observations: three samples, two replicates ([1]Az adatok 6 mérés – 3 párhuzamos x 2 ismétlés – pH-átlagát jelölik).

8. táblázat: 3 g/L Chlorella vulgaris *biomassza hatása* Enterococcus faecium *savtermelésére[1] 12% szárazanyag-tartalmú tej-tápközegben*

Idő, óra(1), Kontroll(2), Chlorell*ával kiegészített(3)*

Table 9

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

Time, h (1)	**Control (2)**	***Chlorella*-supplemented (3)**
0	6.78[a]	6.88[a]
8	8.18[b]	8.52[a]
12	8.31[b]	8.92[a]
22	8.61[b]	8.98[a]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) ($P < 0.05$).
[1]Values are log cfu/mL means, based on six observations: three samples, two replicates ([1]Az adatok 6 vizsgálat – 3 párhuzamos x 2 ismétlés – log cfu/mL-átlagát jelölik).

9. táblázat: 3 g/L Chlorella vulgaris biomassza hatása Lactobacillus plantarum szaporodására[1] 12% szárazanyag-tartalmú tej-tápközegben

Idő, óra(1), Kontroll(2), Chlorellá*val kiegészített(3)*

Table 10

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

Time, h (1)	**Control (2)**	***Chlorella*-supplemented (3)**
0	6.83[a]	6.93[a]
8	8.26[b]	8.66[a]
12	8.41[b]	8.96[a]
22	8.72[b]	9.08[a]

[a,b]Means within a row without a common superscript differ ([a,b]Az egyazon sorban szereplő, eltérő betűjelzésű átlagok szignifikánsan eltérnek egymástól) (*P* < 0.05).
[1]Values are log cfu/mL means, based on six observations: three samples, two replicates ([1]Az adatok 6 vizsgálat – 3 párhuzamos x 2 ismétlés – log cfu/mL-átlagát jelölik).

10. táblázat: 3 g/L Chlorella vulgaris biomassza hatása Enterococcus faecium szaporodására[1] 12% szárazanyag-tartalmú tej-tápközegben

Idő, óra(1), Kontroll(2), Chlorellá*val kiegészített(3)*