

Effect of green alga *Planktochlorella nurekis* on selected bacteria revealed antibacterial activity *in vitro*

L. ČERMÁK¹, Š. PRAŽÁKOVÁ¹, M. MAROUNEK^{1,2}, M. SKŘIVAN¹, E. SKŘIVANOVÁ^{1,2}

¹Institute of Animal Science, Prague-Uhřetěves, Czech Republic

²Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic

ABSTRACT: The green alga *Planktochlorella nurekis* (Chlorellaceae, Chlorophyta) is considered a producer of antibacterial mixture of long-chain fatty acids, which has possibly similar composition and mode of action as chlorellin produced by another green alga, *Chlorella vulgaris*. Although the antibacterial properties of *C. vulgaris* have been reported, the interactions of *P. nurekis* with bacteria have not been determined yet. The aim of this study was to elucidate the effect of *P. nurekis* water suspension on growth of selected gastrointestinal bacteria *in vitro* so that it could be used as a suitable feed supplement in animal farming. Unknown bacterial populations occurring in the algal suspension were identified using 16S rRNA sequencing assay. Selected strains were cultivated with lyophilized *P. nurekis* and the antibacterial effect was monitored. The composition of fatty acids and heat sensitivity of antibacterial substances were also examined. Sequencing analysis of 71 bacterial 16S rRNA genes in xenic algal suspension identified common environmental microbiota, one strain belonging to the class Alphaproteobacteria, 17 to Betaproteobacteria, 44 to Gammaproteobacteria (dominated by *Pseudomonas putida* strains), and nine to Sphingobacteria. The antimicrobial activity of *P. nurekis* suspension was tested at a concentration range of 0.75–6 mg/ml. The highest inhibitory effect was observed on bifidobacteria. Statistically significant reductions in bacterial counts were also observed for *Escherichia coli*, *Salmonella enterica* var. Enteritidis, *S. enterica* var. Infantis, *Campylobacter jejuni*, and *Arcobacter butzleri*. The growth of *Lactobacillus johnsonii* was significantly stimulated. The relative proportions of C14–C22 fatty acids in *P. nurekis* were found as follows: saturated 54.28%, monounsaturated 30.40%, and polyunsaturated 7.16%. The antibacterial compounds present in *P. nurekis* suspension exhibited thermostability. The results indicate that *P. nurekis* can inhibit some pathogenic gastrointestinal bacteria and seems to be a promising essential nutrients source in animal nutrition.

Keywords: chlorellin; fatty acids; gastrointestinal bacteria; interaction; microalgae; thermostability

INTRODUCTION

Unicellular green algae have shown promising antibacterial properties in recent years (Prakash et al. 2011). Among representatives of the Chlorophyta phylum, the strongest antibacterial activity was observed for *Chlorella* spp. (Ordog et al. 2004). *Chlorella vulgaris* has been studied using a variety of practical approaches, and a number of

experiments in mice and rats have shown its protective effect against bacterial and viral infections (Hasegawa et al. 1994). The protective effect of *C. vulgaris* against tumours has also been reported (Justo et al. 2001). Various *Chlorella* strains have been exploited for their health-promoting effects and have been used also as food additives, nutrition in aquaculture, and in the cosmetic industry (Spolaore et al. 2006).

Supported by the Ministry of Agriculture of the Czech Republic (Project MZeRO0714) and by the Internal Grant Agency of the Czech University of Life Sciences Prague (CIGA) (Project No. 20142014).

Algae produce a number of secondary metabolites as chemical defences against predation, herbivory, and competition for space (De Lara-Isassi et al. 2000). For example, *C. vulgaris* produces chlorellin, a mixture of fatty acids (FAs), which expresses antibacterial properties (Pratt et al. 1942; Robles Centeno and Ballentine 1999). In the 1940s, Pratt and co-workers observed that growth inhibition was destroyed by boiling, and found out the substance is water-soluble and capable of passing through the cell membrane. This is contrary to the later studies (e.g. Fergola et al. 2007), which found out that chlorellin is mainly composed of hydrophobic C18 FAs.

A green alga, *Planktchlorella nurekis* (PN), has recently been defined as a new genus and species belonging to the *Parachlorella* clade in Chlorellaceae, Chlorophyta (Skaloud et al. 2014). This strain, formerly *C. vulgaris* IFR C-111, originates from Nurek Dam, Tajikistan, where it was originally isolated in 1977, and also occurs in Kazinga Channel, Uganda. It is characterized by a reduced sedimentation rate and a rather wide temperature tolerance (Skaloud et al. 2014). PN is a unicellular autotrophic non-sedimenting alga with uninuclear, spherical, planktonic vegetative cells. This genus differs from the other genera of the family by the order of the nucleotides in its unique SSU and ITS 18S rRNA gene sequences (100% identity for SSU; 99.4% identity for ITS) (Skaloud et al. 2014). The authentic strain CAUP H 8701 has recently been cryopreserved in a metabolic inactive state at the Culture Collection of Algae of Charles University in Prague, Czech Republic (Skaloud et al. 2014).

Currently more than 30 000 t dry weight of microalgae are produced commercially every year. Algae are typically first thought of as a source of dietary protein (Becker 2007), in *C. vulgaris* it is 51–58%. Algae also contain long-chain polyunsaturated fatty acids, fytosterols, carbohydrates, and vitamins. *C. vulgaris*-based feed supplements have been tested in production animals with positive results. *Chlorella* spp. were tested on the entire microbial community in the gastrointestinal tract (GIT) of laying hens (Janczyk et al. 2009), which showed significantly increased productivity and egg quality by long-term feeding *C. vulgaris*. The application of the C-111 strain during pig fattening also reduced the need for veterinary chemotherapeutic agents (Chervanov et al. 2011). It suggests itself that PN could be, just as *C. vul-*

garis, a suitable feed supplement in farm animal production. However, there is limited knowledge of its interactions with bacteria and no information regarding the microbial composition of the algal suspension itself.

The purpose of this study was to assess the future use of PN in animal feed, which could benefit from high content of proteins, lipids, antioxidants, and also from possible pathogen reduction. In particular, the aims were following: first to identify the bacteria present in PN suspension using the analysis of bacterial 16S rRNA genes, in order to estimate possible contribution of these bacteria to antibacterial activity against the tested strains, and to confirm safety of PN suspension for potential use as a feed supplement. Second goal was to evaluate the antibacterial properties of PN suspension against selected probiotic bacteria or bacteria inhabiting GIT of animals. Third goal was focused on the FA composition of PN suspension. The antibacterial effect was attributed to chlorellin and its thermostability was tested to confirm its lipidic nature.

MATERIAL AND METHODS

Algal strain. The algal suspension of *P. nurekis* CAUP H 8701 (= 1904 KIEG) was provided by Key Industry Engineering Group (KIEG, Prague, Czech Republic), where it is cultivated under specific conditions in a defined liquid medium based on cellulose residues, ammonium nitrate, and carbon dioxide. It grows in the artificial light provided by 250 W lamps, lighting lasts 10–12 h a day. Because of its activity against bacteria, fungi, and blue-green algae, it is not necessary to cultivate the algal suspension under sterile conditions (US Patent No. US2012/0225036A1).

Lyophilization of algal suspension. The lyophilization was performed using the Christ Alpha 2-4 LSC Freeze Dryer (Pragolab, Prague, Czech Republic). The algal suspension (3 l) was split into aliquots in polypropylene bowls, frozen at –80°C, and lyophilized for 60 h at increasing temperature (sequentially –10, 0, 10, 20, and 30°C, each for 12 h) under low pressure.

PCR amplification of 16S rRNA genes. DNA from 100 mg of lyophilized PN was extracted with a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) and used as the template for 16S rRNA gene amplification. To identify the bacteria in the algal suspension, degenerate prim-

doi: 10.17221/8522-CJAS

ers 16Seu27f (5'AGAGTTTGGATCMTGGCKCAG) (Lane 1991) and 16Seu783r (an equimolar mix of 5'CTACCAGGGTATCTAATCCTG, 5'CTACCGGGTATCTAATCCCG, and 5'CTACCCGGG-TATCTAATCCGG) (Sakai et al. 2004) were used. The PCR mixture contained 50 ng DNA, 0.25 mM of each primer, 50 mM of each dNTP, and 1 U LA polymerase (Top-Bio, Prague, Czech Republic) in a total volume of 50 ml. The PCR amplification consisted of the following steps: an initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 45 s, annealing at 56°C for 30 s, and extension at 68°C for 30 s; and a final extension step at 68°C for 5 min.

Cloning and sequencing. The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, cloned into the pGEM-T vector (Promega, Madison, USA), and sequenced. The sequences of 71 clones were obtained by capillary sequencing using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, USA) by Macrogen Inc. (Amsterdam, the Netherlands). The sequences were assigned to existing taxa using the Ribosomal Database Project (RDP) classifier tool (Wang et al. 2007) and checked for chimeras using Bellerophon web tool (Huber et al. 2004).

Bacterial strains. Representative species of enteropathogenic bacteria, food-borne pathogens, and some probiotic bacteria were used. The following strains were used for *in vitro* assays: *Escherichia coli* ATCC 29522, *Salmonella enterica* var. Enteritidis ATCC 13076, *Campylobacter jejuni* CCM 6214 (ATCC 33560), *Clostridium perfringens* CCM 4435 (ATCC 13124), *Lactobacillus acidophilus* CCM 4833, *Lactobacillus johnsonii* CCM 4384, and *Arcobacter butzleri* CCM 7051 (Czech Collection of Microorganisms, Brno, Czech Republic); *E. coli* E22 (clinical isolate provided by Dr. A. Milon from National Veterinary College, Toulouse, France); *S. enterica* var. Typhimurium K2, *S. enterica* var. Infantis K3 (Institute of Chemical Technology, Prague, Czech Republic); *C. jejuni* VFU 6189 (a kind gift from Dr. I. Steinhäuserová from the University of Veterinary Sciences, Brno, Czech Republic); *C. perfringens* CNCTC 5459 (Czechoslovak National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic); and *Bifidobacterium bifidum* JKM and *Bifidobacterium longum* TP1 (provided by Dr. E. Vlková from the Czech University of Life Sciences, Prague, Czech Republic).

Antibacterial assay. The bacteria were cultivated in triplicate using media with different concentrations of lyophilized PN suspension. For each bacterial strain, a set of fifteen (3 × 5) 10-ml flasks, each containing 2.5 ml of 2 × concentrated medium, were closed with rubber stoppers and autoclaved at 120°C for 15 min. A PN stock suspension (20 g/l) was serially diluted to concentrations of 6, 3, 1.5, and 0.75 g/l, and the sterile media in twelve of the flasks were supplemented with diluted PN using sterile needles. Three flasks contained no algae. All flasks were inoculated with the respective bacterial strain (0.3 ml), and the volumes were adjusted with sterile water to 5 ml. The flasks used for anaerobes were filled with CO₂. The flasks with media used for microaerophilic bacteria were filled with the mixture of 85% N₂, 10% CO₂, and 5% O₂.

The cultures were grown still (no shaking) at 37°C for 24 h, except for bifidobacteria, lactobacilli, and both *C. jejuni* strains, which were cultivated for 48 h. The cultures were then diluted into a tenfold dilution series, plated on agar media in Petri dishes, and cultivated under appropriate conditions for 1–3 days. Countable numbers of colony forming units (CFU) were counted and statistically evaluated. The exact conditions of cultivation for each bacterium, such as broth composition, agar medium, supplements, or plating technique, are listed in Table 1. Glucose broth (GB) comprised glucose 5 g/l, bactopectone 6 g/l, yeast extract 3 g/l, KOH 4.6 g/l, NaHCO₃ 3 g/l, (NH₄)₂SO₄ 2.8 g/l, NaCl 1 g/l, MgSO₄·7H₂O 0.1 g/l, Ca(OH)₂ 0.1 g/l, H₃PO₄ 0.36% (v/v). Final concentrations of following ingredients were used: L-cysteine 0.5 g/l, Tween 80 1 ml/l, soya peptone 5 g/l. All media and supplements were supplied by Oxoid, Brno, Czech Republic.

Fatty acid analysis. Lipids extracted from 20 mg of lyophilized algal suspension were obtained by chloroform-methanol extraction (2 : 1, v/v). The air-dried lipidic extract was mixed with 3 ml 0.5M NaOH in methanol and incubated at 50°C for 30 min, then 2 ml HCl (35%, w/w) was added and the mixture was further incubated at 50°C for 30 min to form FA methyl esters. Subsequently, 4 ml hexan and water (1 : 1) was added, intensely mixed, and centrifuged at 1200 g for 5 min. The lower (organic) phase was dissolved in 1 ml hexan. One microlitre of the sample was injected into a gas chromatograph Agilent Technologies 6890 N (Agilent Technologies, Santa Clara, USA) for FA

Table 1. Cultivation conditions of gastrointestinal tract microbiota representatives

Bacterium	Liquid broth + supplements	Agar medium + supplements	Plating technique	Cultivation conditions on agar plates
<i>Escherichia</i>	GB	MacConkey agar No.3.	spread	aerobic, 37°C, 24 h
<i>Salmonella</i>	GB	X.L.D. agar	spread	aerobic, 37°C, 24 h
<i>Arcobacter</i>	Arcobacter Broth + CAT	Mueller-Hinton agar	spread	aerobic, 37°C, 48 h
<i>Campylobacter</i>	Nutrient Broth No. 2	Campylobacter Agar Base + PCSS + CGS + LHB	spread	microaerophilic, 37°C, 72 h
<i>Clostridium</i>	GB + L-cysteine	Perfringens Agar Base + EYE + TSC	pour	anaerobic, 37°C, 24 h
<i>Bifidobacterium</i>	Wilkins-Chalgren Anaerobe Broth + SP + Tween 80 + L-cysteine + mupirocin (50 mg/l)	Wilkins-Chalgren Anaerobe Agar + SP + Tween 80 + L-cysteine	pour	anaerobic, 37°C, 72 h
<i>Lactobacillus</i>	M.R.S. broth	M.R.S. agar	pour	aerobic, 37°C, 72 h

GB = glucose broth, CAT = C.A.T. Selective Supplement, PCSS = Preston Campylobacter Selective Supplement, CGS = Campylobacter Growth Supplement, LHB = Laked Horse Blood, EYE = Egg Yolk Emulsion, TSC = T.S.C. Selective Supplement, SP = Soya Peptone

methyl ester analysis. FA methyl esters were identified by co-chromatography with authentic standards (Sigma-Aldrich, Prague, Czech Republic).

Thermostability of PN antibacterial compounds. The thermostability of the active compounds from PN was examined by a cultivation experiment, for which the representative strain *E. coli* ATCC 29522 and the highest concentration of lyophilized PN 6 g/l were used. The experiments were performed in triplicate. Four temperatures (60, 80, 100, and 120°C) for 30 min and three periods (60, 120, and 180 min) of exposure to 105°C were assessed to identify the limit of the antibacterial effect. After cooling to 37°C, the heat-treated PN was added to flasks with medium, as described above, and *E. coli* was inoculated. Two types of controls were used: one containing *E. coli* alone and the second comprising *E. coli* and heat-untreated PN.

Statistical assay. A one-way analysis of variance with multiple comparisons (Scheffé's method) was used to evaluate statistically significant differences among the decadal logarithms of the CFU counts according to the algal concentrations. The data analysis was performed with SAS software (Statistical Analysis System, Version 8.2, 2001).

RESULTS

Composition of the xenic algal suspension. The sequencing analysis of 71 bacterial 16S rRNA genes in the PN suspension identified common water and soil microbiota, including Betaproteobacteria (class Comamonadaceae; 17 sequences), Gammapro-

teobacteria (44 sequences corresponding to *Pseudomonas putida*), Sphingobacteria (9 sequences), and Alphaproteobacteria (1 sequence) (Table 2). The sequences are available online at the NCBI database under Accession No. KF020798–KF020868.

Effects of the algal suspension on representatives of GIT microbiota. The antibacterial assay revealed interactions between the algal suspension and bacterial strains. Out of 14 GIT microbiota representatives, 9 strains were significantly influenced by PN suspension *in vitro* ($P < 0.05$) (Table 3). The most remarkable effect occurred on *B. bifidum* JKM and *B. longum* TP1. Incubation with the highest concentration of PN (6 g/l) reduced the counts of bifidobacteria by 2.82–2.97 log₁₀ CFU/ml compared with the positive control.

The incubation of *C. jejuni* CCM 6214 and *C. jejuni* VFU 6189 with 3 g/l and 6 g/l of PN led to a significant reduction in bacteria with 0.5–1.17 log₁₀ CFU/ml as compared to control. Viable counts of *E. coli* ATCC 29522, *E. coli* E22, *S. enterica* var. Enteritidis, and *S. enterica* var. Infantis were significantly reduced by one or two orders of magnitude with 6 g/l of PN treatment. The growth suppression of *A. butzleri* with 6 g/l PN was moderate but still significant. In contrast, a stimulating effect was observed for *L. johnsonii*, with CFU counts increasing with the increasing algal concentration. However, no effect was observed on *L. acidophilus*, *S. enterica* var. Typhimurium or *C. perfringens*.

Fatty acid composition of PN. The composition of C14–C23 FAs in the PN dried suspension was determined (Table 4). The predominant fraction

doi: 10.17221/8522-CJAS

Table 2. List of 71 sequences assigned to taxonomic groups and genera by RDP classifier (at 80% threshold) and proportional representation of bacterial classes

Class	Family	Genus	Number of sequences	Percentage of bacterial class
<i>Alphaproteobacteria</i>	<i>Acetobacteraceae</i>	<i>Roseomonas</i>	1	1.4
	<i>Comamonadaceae</i>	<i>Acidovorax</i>	8	23.9
<i>Betaproteobacteria</i>	<i>Rhodocyclaceae</i>	<i>Caenimonas</i>	1	
		<i>Comamonas</i>	2	
		<i>Curvibacter</i>	2	
		<i>Hydrogenophaga</i>	1	
		<i>Methyloversatilis</i>	2	
		<i>Zoogloea</i>	1	
<i>Gammaproteobacteria</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>	44	62.0
	<i>Cyclobacteriaceae</i>	<i>Algoriphagus</i>	5	12.7
<i>Sphingobacteria</i>	<i>Cytophagaceae</i>	<i>Runella</i>	1	
	<i>Chitinophagaceae</i>	<i>Sediminibacterium</i>	3	
Total sequences			71	100.0

consisted of saturated FAs (palmitic, stearic, and myristic acids), 54.28% of the total FA content, followed by monounsaturated FAs (oleic and palmitoleic acids) at 30.40%, and polyunsaturated FAs (linoleic, α -linolenic, and γ -linolenic acid) at 7.16% of the total FA content. The C18 FAs represented 62.28% of all the detected FAs, with a predomination of oleic and stearic acids.

Thermostability of PN antibacterial compounds. In this study, the exposure of a PN suspension to 60, 80, 100, and 120°C for 30 min did

not show any significant difference in the PN antibacterial effect in comparison to the unheated control ($P = 0.063$) (Figure 1A). It was also found that the duration of exposure to 105°C had no effect on the antimicrobial activity of PN ($P = 0.441$) (Figure 1B).

The antibacterial activity against *E. coli* ATCC 29522 was confirmed by a thermostability experiment (Figure 1). There was a significant reduction in *E. coli* CFUs in all flasks containing treated or untreated alga ($P < 0.001$).

Table 3. Effect of lyophilized *P. nurekis* (0.75–6 g/l) on gastrointestinal tract microbiota (Log_{10} CFU/ml) in the cultivation experiment. All counts were performed in triplicates on selective media, values are means with standard deviations. Values at the same row with different superscripts are significantly different ($P < 0.05$)

Bacterial strain	Control	0.75 g/l	1.5 g/l	3 g/l	6 g/l
<i>E. coli</i> ATCC 29522	9.55 \pm 0.14 ^{ab}	9.67 \pm 0.04 ^b	9.30 \pm 0.02 ^c	9.39 \pm 0.02 ^{bc}	7.23 \pm 0.02 ^d
<i>E. coli</i> E22	9.13 \pm 0.07 ^a	9.03 \pm 0.04 ^a	9.21 \pm 0.03 ^a	9.00 \pm 0.11 ^a	7.83 \pm 0.15 ^b
<i>S. enterica</i> var. Enteritidis ATCC 13076	8.99 \pm 0.05 ^a	8.67 \pm 0.25 ^a	8.86 \pm 0.24 ^a	8.57 \pm 0.27 ^a	7.72 \pm 0.23 ^b
<i>S. enterica</i> var. Infantis K2	9.02 \pm 0.04 ^a	9.21 \pm 0.08 ^a	9.17 \pm 0.19 ^a	9.17 \pm 0.06 ^a	8.49 \pm 0.27 ^b
<i>S. enterica</i> var. Typhimurium K3	8.99 \pm 0.13	9.10 \pm 0.25	8.85 \pm 0.11	8.59 \pm 0.36	8.59 \pm 0.19
<i>C. jejuni</i> CCM 6189	8.94 \pm 0.05 ^a	8.89 \pm 0.15 ^a	8.57 \pm 0.27 ^a	7.77 \pm 0.10 ^b	7.81 \pm 0.15 ^b
<i>C. jejuni</i> 6214	9.31 \pm 0.09 ^a	9.46 \pm 0.09 ^a	9.32 \pm 0.21 ^a	8.81 \pm 0.22 ^b	8.61 \pm 0.14 ^b
<i>A. butzleri</i> CCM 7051	7.92 \pm 0.05 ^{ab}	8.18 \pm 0.14 ^a	7.90 \pm 0.13 ^{ab}	7.99 \pm 0.11 ^{ab}	7.80 \pm 0.14 ^b
<i>L. johnsonii</i> 4384	8.49 \pm 0.09 ^c	8.63 \pm 0.02 ^{bc}	8.71 \pm 0.07 ^{ab}	8.76 \pm 0.03 ^{ab}	8.82 \pm 0.02 ^a
<i>L. acidophilus</i> 4833	8.44 \pm 0.06	8.67 \pm 0.08	8.48 \pm 0.11	8.62 \pm 0.13	8.73 \pm 0.10
<i>C. perfringens</i> CCM 4435	7.67 \pm 0.05	7.81 \pm 0.08	7.61 \pm 0.08	7.60 \pm 0.15	7.70 \pm 0.11
<i>C. perfringens</i> CNCTC 5459	7.68 \pm 0.11	7.83 \pm 0.19	7.71 \pm 0.06	7.77 \pm 0.19	7.64 \pm 0.05
<i>B. bifidum</i> JKM	8.07 \pm 0.22 ^a	7.82 \pm 0.20 ^a	8.15 \pm 0.13 ^a	4.75 \pm 0.39 ^b	5.10 \pm 0.28 ^b
<i>B. longum</i> TP1	8.60 \pm 0.05 ^a	7.31 \pm 0.15 ^b	7.24 \pm 0.04 ^b	6.14 \pm 0.27 ^c	5.78 \pm 0.33 ^c

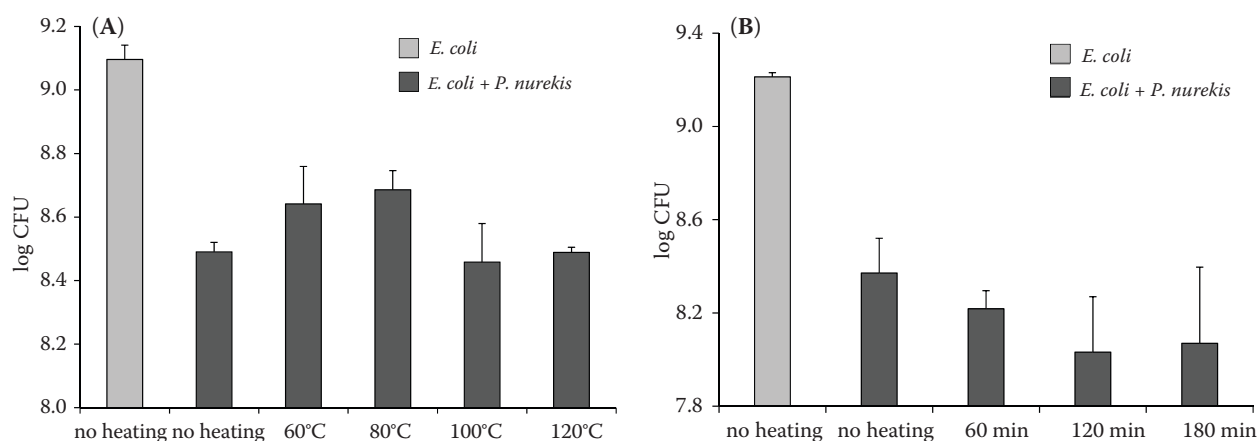


Figure 1. Assessing thermostability of the active compounds in lyophilized *P. nurekis* (6 g/l) on *E. coli* ATCC 29522 (Log_{10} CFU/ml including standard deviation) in the cultivation experiment after various heating treatments conducted just before the *E. coli* inoculation

(A) temperature range 60–120°C for 30 min, (B) time of exposure to 105°C

DISCUSSION

Green microalgae have been studied using a variety of practical approaches, and there have been many reports demonstrating the protective effects of these algae against diseases and ailments, e.g. bacterial and viral infections, tumours, and peptic ulcers, in recent years. Antimicrobial properties of green algae are considered to be an effective alternative to antibiotic growth promoters in the diets to maintain optimum health and productivity of the production animals (poultry, cattle, sheep, pigs). From a technological point of view, one of the greatest disadvantages of using microalgae suspension is sedimentation. For human food supplements, this problem is avoided by producing tablets or capsules from the microalgal suspension. However, this process is cost-prohibitive for animal nutrition, and it affects the microalgal nutritional values and possibly their antibacterial properties (Komaki et al. 1998). If the biomass is to be fed to animals, the cell walls

of most species of microalgae must be ruptured to achieve maximum digestibility of the algae. The most practical methods of processing algae are generally mechanical or thermal, which can be achieved with drum drier, spray drier, direct cooking, boiling or steam injection. For instance, a positive effect of spray-dried *C. vulgaris* fed to laying hens was observed by Englmaierova et al. (2013), the alga increased the concentration of health-promoting carotenoids in yolks. Moreover, the use of natural algae corresponds with standards of organic farming. Our study provides information on the antibacterial effects of *Planktochlorella nurekis* CAUP H 8701, which might have, just as *C. vulgaris*, a promising beneficial use in the fields of human and animal nutrition.

A fundamental complication of the entire study was the use of a xenic algal suspension. Since it was not possible to gain a sterile culture of algae, the composition of the algal suspension was assayed. The most frequent contaminating bacteria, though still a small fraction, were identified as

Table 4. C14–C23 fatty acids (FA) composition of the *P. nurekis* biomass. Only FAs amounting over 0.5% of the total FA content are shown

FA	Scheme	Total FA (%)	FA	Scheme	Total FA (%)	FA	Scheme	Total FA (%)
Myristic	14:0	1.74	oleic	18:1n9	28.37	arachic	20:0	0.71
Palmitic	16:0	26.91	<i>cis</i> -vaccenic	18:1n7	1.13	eicosadienoic	20:2-n6	0.63
Palmitoleic	16:1n7	2.03	linoleic	18:2-n6	4.13	eicosatrienoic	20:3-n3	1.86
Margaric	17:0	0.53	α -linolenic	18:3-n3	0.87	erucic	22:1-n9	1.27
Stearic	18:0	25.62	γ -linolenic	18:3-n6	2.17	tricosylic	23:0	1.47

doi: 10.17221/8522-CJAS

Gram-negative bacteria belonging to Betaproteobacteria/Comamonadaceae and Gammaproteobacteria/Pseudomonadaceae taxa. The bacterial class Comamonadaceae are aerobic, motile, and ubiquitous in the environment and have been isolated from soil, mud, and water. *Comamonas* strains have also been isolated from denitrifying activated sludge as well as from various clinical samples and from the hospital environment, but they are not considered to be pathogenic to healthy humans or animals (Willems and De Vos 2006). The bacteria of class Pseudomonadaceae found in the algal culture were represented by only one species, *Pseudomonas putida* (over one half of all the identified species). *P. putida* is a rod-shaped, flagellated, Gram-negative bacterium that is found in most soil and water habitats with sufficient oxygen. *P. putida* has demonstrated potential biocontrol properties as an effective antagonist of damping off disease caused by *Fusarium* (Validov et al. 2007). Infections caused by *P. putida* are rare and are mostly reported in immunocompromised patients (Manfredi et al. 2000). A contribution of contaminating bacteria in PN suspension to inhibition of some tested bacteria cannot be excluded, but if there was any, it would have been very likely with negligible and rather unharmed effects.

Although there are many studies confirming the susceptibility of various *E. coli* strains to organic extracts of microalgae (e.g. Demiriz et al. 2011), some have reached opposite conclusions (Kellam and Walker 1989). In accordance with our results, an organic solvent extract from *C. vulgaris* was found to be effective against *S. enterica* var. Enteritidis ATCC 13076 (Demiriz et al. 2011). Waldenstedt et al. (2003) studied the antibacterial effect of the carotenoid astaxanthin produced by the microalga *Haematococcus pluvialis* (Chlorophyceae) on *C. jejuni* and *C. perfringens* in the GIT of chickens. An *in vivo* experiment reported that the counts of *C. jejuni* remained unchanged and that the counts of *C. perfringens* decreased significantly (Waldenstedt et al. 2003); however, our *in vitro* findings were the opposite.

Recently, Beheshtipour et al. (2012) have observed that the presence of *C. vulgaris* significantly increases the viability of *L. acidophilus* LA-5. Although we did not observe such a phenomenon for *L. acidophilus* CCM 4833, moderate growth stimulation was observed in our experiments for *L. johnsonii* CCM 4384 cultivated with 0.75–6 g/l

of *P. nurekis*. Additionally, Rasmussen et al. (2009) observed a reduction on agar media in lactobacilli CFU from the colon of mice fed a diet with the blue-green alga *Nostoc commune*. According to Safonova and Reisser (2005), some microalgae might be able to promote or inhibit the growth of heterotrophic bacteria. Both microorganisms can act as competitors (e.g. for carbon sources), or microalgae can stimulate bacterial growth by providing increased nutrients for lactic acid bacteria and probiotic bacteria (Beheshtipour et al. 2012).

The FA composition of PN showed a dominance of C16 and C18 chain length FAs, which could be expected for *Chlorella* spp. and other green algae (Villar-Argaiz et al. 2009; Griffith et al. 2011). The mode of action of FAs on target cells is not completely known, however, Klausner et al. (1980) have suggested that they can severely damage the biological membranes of eukaryotic cells. Nevertheless, such action is distinctively limited to free FAs, which are not bound in lipids. Pribyl et al. (2012) defined the FA composition of *C. vulgaris* CICALA 256 as a mixture of polyunsaturated linoleic and α -linolenic acids (43.93% of the total FA content), saturated palmitic, stearic, and myristic acids (29.10%), and monounsaturated oleic and palmitoleic acids (19.78%). In contrast to their results, the level of polyunsaturated FAs in PN was markedly lower. In addition, Fergola et al. (2007) prepared synthetic chlorellin as a mixture of four C18 FAs (stearic, oleic, linoleic, and linolenic acids) and assayed the impact on another green alga, *Pseudokirchneriella subcapitata* (newly *Raphidocelis subcapitata*). The results of that study suggest the use of artificial chlorellin, available in large amounts, instead of natural chlorellin, which is produced in limited quantities (Fergola et al. 2007). Still, some additional antibacterial effect of other compounds present in PN, e.g. polyphenols, cannot be excluded and deserve to be examined in future assays.

The results on thermostability of the active substances produced by PN proved our hypothesis. Pratt (1942) stated that antibacterial effect of *C. vulgaris* is destroyed by heat. However, we did not find the same conclusion in our study. The active substances produced by PN appear to be heat-stable up to 120°C. Furthermore, linoleic acid, a major fraction of chlorellin, as well as other FAs, were found to be stable at a temperature of 300°C or below (Shin et al. 2012).

CONCLUSION

The antibacterial activity of microalga *Planktochlorella nurekis* against GIT microbiota representatives was studied. The identification of bacterial genera in the xenic microalgal suspension revealed the presence of common fresh-water health-safe microbiota. A significant growth suppression was detected for *B. bifidum*, *B. longum*, *C. jejuni*, *S. enterica* var. *Enteritidis*, *S. enterica* var. *Infantis*, *A. butzleri*, and *E. coli*, whereas the growth of *L. johnsonii* was slightly stimulated. The active compounds present in the *Planktochlorella nurekis* suspension exhibited thermostability, which corresponded to our expectations that the active compounds were of a lipidic nature. The results of this study may help provide essential knowledge on utilizing green microalgae as suitable alternative to in-feed antibiotics to prevent diseases in animal husbandry and to maintain the microbial safety of animal products.

Acknowledgement. The authors thank E. Vlková, I. Steinhauserová, and A. Milon for providing aforementioned bacterial strains and V. Vejskalová for performing the gas chromatography.

REFERENCES

- Becker E.W. (2007): Micro-algae as a source of protein. *Biotechnology Advances*, 25, 207–210.
- Beheshtipour H., Mortazavian A.M., Haratian P., Darani K.K. (2012): Effects of *Chlorella vulgaris* and *Arthrospira platensis* addition on viability of probiotic bacteria in yogurt and its biochemical properties. *European Food Research and Technology*, 235, 719–728.
- Chervanev V.A., Tarasenko P.A., Petrova Z.G. (2011): *Chlorella*: improving reserve capacity in pig production. *Svinovodstvo*, 1, 38–41.
- De Lara-Isassi G., Alvarez-Hernandez S., Collado-Vides L. (2000): Ichthyotoxic activity of extracts from Mexico marine microalgae. *Journal of Applied Phycology*, 12, 45–52.
- Demiriz T., Cokmus C., Pabuccu K. (2011): Antimicrobial activity of some algal species belonging to Cyanobacteria and Chlorophyta source. *Asian Journal of Chemistry*, 23, 1384–1386.
- Englmaierova M., Skrivan M., Bubancova I. (2013): A comparison of lutein, spray-dried *Chlorella*, and synthetic carotenoids effects on yolk colour, oxidative stability, and reproductive performance of laying hens. *Czech Journal of Animal Science*, 58, 412–419.
- Fergola P., Cerasuolo M., Pollio A., Pinto G., Della Greca M. (2007): Allelopathy and competition between *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*: experiments and mathematical model. *Ecological Modelling*, 208, 205–214.
- Griffith M.J., Van Hille R.P., Harrison S.T.L. (2011): Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions. *Journal of Applied Phycology*, 24, 989–1001.
- Hasegawa T., Okuda M., Nomoto K., Yoshikai Y. (1994): Augmentation of the resistance against *Listeria monocytogenes* by oral administration of a hot water extract of *Chlorella vulgaris* in mice. *Immunopharmacology and Immunotoxicology*, 16, 191–202.
- Huber T., Faulkner G., Hugenholtz P. (2004): Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics*, 20, 2317–2319.
- Janczyk P., Halle B., Souffrant W.B. (2009): Microbial community composition of the crop and ceca contents of laying hens fed diets supplemented with *Chlorella vulgaris*. *Poultry Science*, 88, 2324–2332.
- Justo G.Z., Silva M.R., Querioz M.L.S. (2001): Effects of the green algae *Chlorella vulgaris* on the response of the host hematopoietic system to intraperitoneal Ehrlich ascites tumor transplantation in mice. *Immunopharmacology and Immunotoxicology*, 23, 119–132.
- Kellam S.J., Walker J.M. (1989): Antibacterial activity from marine microalgae in laboratory culture. *British Phycological Journal*, 24, 191–194.
- Klausner R.D., Kleinfeld A.M., Hoover R.L., Karnovsky M.J. (1980): Lipid domains in membranes. Evidence derived from structural perturbations induced by free fatty acids and lifetime heterogeneity analysis. *Journal of Biological Chemistry*, 255, 1286–1295.
- Komaki H.M., Yamashita Y., Niwa Y., Tanaka N., Kamiya Y.A., Furuse M. (1998): The effect of processing of *Chlorella vulgaris*: K-5 on in vitro and in vivo digestibility in rats. *Animal Feed Science and Technology*, 70, 363–366.
- Lane D. (1991): 16S/23S rRNA sequencing. In: Stackebrandt E. and Goodfellow M. (eds): *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, Chichester, UK, 115–175.
- Manfredi R., Nanetti A., Ferri M., Chiodo F. (2000): *Pseudomonas* spp. complications in patients with HIV disease: an eight-year clinical and microbiological survey. *European Journal of Epidemiology*, 16, 111–118.
- Ordog V., Stirk W.A., Lenobel R., Bancirova M., Strnad M., van Staden J., Szigeti J., Nemeth L. (2004): Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. *Journal of Applied Phycology*, 16, 309–314.

doi: 10.17221/8522-CJAS

- Prakash J.W., Antonisamy J.M., Jeeva S. (2011): Antimicrobial activity of certain fresh water microalgae from Thamirabarani River, Tamil Nadu, South India. *Asian Pacific Journal of Tropical Biomedicine*, 1, 170–173.
- Pratt R. (1942): Studies on *Chlorella vulgaris*. V. Some properties of the growth-inhibitor formed by *Chlorella* cells. *American Journal of Botany*, 29, 142–148.
- Pribyl P., Cepak V., Zachleder V. (2012): Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris*. *Applied Microbiology and Biotechnology*, 94, 549–561.
- Rasmussen H.E., Martinez I., Lee J.Y., Walter J. (2009): Alteration of the gastrointestinal microbiota of mice by edible blue-green algae. *Journal of Applied Microbiology*, 107, 1108–1118.
- Robles Centeno P.O., Ballentine D.L. (1999): Effects of culture conditions on production of antibioticly active metabolites by the marine alga *Spyridia filamentosa* (Ceramiaceae, Rhodophyta). I. Light. *Journal of Applied Phycology*, 11, 217–224.
- Safonova E., Reisser W. (2005): Growth promoting and inhibiting effects of extracellular substances of soil microalgae and cyanobacteria on *Escherichia coli* and *Micrococcus luteus*. *Phycological Research*, 53, 189–193.
- Sakai M., Matsuka A., Komura T., Kanazawa S. (2004): Application of a new PCR primer for terminal restriction fragment length polymorphism analysis of the bacterial communities in plant roots. *Journal of Microbiological Methods*, 59, 81–89.
- Shin H.-Y., Ryu J.-H., Park S.-Y., Bae S.-Y. (2012): Thermal stability of fatty acids in subcritical water. *Journal of Analytical and Applied Pyrolysis*, 98, 250–253.
- Skaloud P., Nemcova Y., Pytela J., Bogdanov N.I., Bock C., Pickinpaugh S.H. (2014): *Planktochlorella nurekis* gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a novel coccoid green alga carrying significant biotechnological potential. *Fottea*, 14, 53–62.
- Spolaore P., Joannis-Cassan C., Duran E., Isambert A. (2006): Commercial Applications of Microalgae. *Journal of Bioscience and Bioengineering*, 101, 87–96.
- Validov S., Kamilova F., Qi S., Stephan D., Wang J.J., Makarova N., Lugtenberg B. (2007): Selection of bacteria able to control *Fusarium oxysporum* f. sp. *radicis-lycopersici* in stonewool substrate. *Journal of Applied Microbiology*, 102, 461–471.
- Villar-Argaiz M., Medina-Sanchez J.M., Bulles J., Delgado-Molina J.A., Perez O.R., Navarro J.C., Carrillo P. (2009): UV radiation and phosphorus interact to influence the biochemical composition of phytoplankton. *Freshwater Biology*, 54, 1233–1245.
- Waldenstedt L., Inbarr J., Hansson I., Elwinger K. (2003): Effects of astaxanthin-rich algal meal (*Haematococcus pluvalis*) on growth performance, caecal campylobacter and clostridial counts and tissue astaxanthin concentration of broiler chickens. *Animal Feed Science and Technology*, 108, 119–132.
- Wang Q., Garrity G.M., Tiedje J.M., Cole J.R. (2007): Native Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, 73, 5261–5267.
- Willems A., De Vos P. (2006): *Comamonas*. In: Dworkin M., Falkow S., Rosenberg E., Schleifer K.-H., Stackebrandt E. (eds): *The Prokaryotes*. Springer-Verlag, New York, USA, 723–736.

Received: 2014–10–03

Accepted after corrections: 2015–05–25

Corresponding Author

Mgr. Ladislav Čermák, Ph.D., Institute of Animal Science, Department of Physiology of Nutrition and Quality of Animal Products, Přátelství 815, 104 00 Prague 10-Uhřetěves, Czech Republic
 Phone: +420 267 009 717, e-mail: cermak.ladislav@vuzv.cz