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Characterization of microcystin-LR removal process in the presence of probiotic bacteria

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ABSTRACT

Toxic cyanobacteria have been reported in lakes and reservoirs in several countries. The presence of toxins in drinking water creates a potential risk of toxin transference for water consumers. Besides chemical and physical methods of cyanotoxin removal from water, biodegradation methods would be useful. The aim of the current study was to identify bacterial removal mechanisms of the hepatotoxin microcystin-LR. This was studied by testing the hypothesis of enzymatic degradation of microcystin-LR in the presence of probiotic lactic acid bacterial and bifidobacterial strains and the participation of the proteolytic system of the bacteria in this process. The results suggest that extracellularly located cell-envelope proteinases are involved in the decomposition of microcystin-LR. In particular, a correlation between proteolytic activity and microcystin removal was found and both these parameters were dependent on glucose as an energy source. In addition, EDTA, which was indicated as a main inhibitor of proteinases of the investigated strain, was shown to limit the rate of microcystin removal. The removal of microcystins was shown to be different from the known microcystin-degradation pathway of *Sphingomonas*. ^{14}C-labeled microcystin was not found inside the cells and bacterial cell extracts were not able to remove the toxin, which supports the involvement of extracellularly located proteinases. The results confirm the hypothesis of enzymatic degradation of microcystins in the presence of probiotic bacteria.

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1. Introduction

Blooms of microcystin-producing cyanobacteria reported worldwide have caused concerns of acute hepatotoxicity while chronic low-level exposure to microcystins has been associated with carcinogenesis and tumor promotion (Ueno et al., 1996; Zhao et al., 2009; Zhou et al., 2002). Therefore, the World Health Organization (WHO) has set the provisional drinking water guideline value at 1.0 μg L^{-1} for microcystin-LR (MC-LR) (WHO, 2004). Human exposure to microcystins occurs mainly through the gastrointestinal tract and these toxins accumulate in the liver as a result of active uptake (Kuiper-Goodman et al., 1999). Possible effects of long-term exposure to low doses of cyanotoxins are still not well known but chronic intoxication with microcystins presented in contaminated water was recognized epidemiologically as a factor responsible for higher prevalence of primary liver cancer in China (Ueno et al., 1996; Yu et al., 2001). Under environmental conditions, microcystins are fairly resistant to different physico-chemical and biological degradation processes, such as high temperature, extreme pH and certain enzymes (Jones and Orr, 1994; Tsuji et al., 1994). Okano et al. (2006) have reported that common enzymes, including proteases, cannot degrade microcystins. Therefore, it is likely that only specific microcystin-degrading organisms can degrade the

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toxins. Previously reported bacteria shown to degrade microcystins include several *Sphingomonas* sp. (Jones and Orr, 1994; Bourne et al., 1996, 2001; Park et al., 2001; Saito et al., 2003; Harada et al., 2004; Valeria et al., 2006), *Pseudomonas aeruginosa* (Takenaka and Watanabe, 1997), *Paucibacter toxinivorans* (Rapala et al., 2005) and *Sphingosinicella microcystinivorans* (Maruyama et al., 2006).

Recently it has also been shown that specific probiotic bacteria provide new means in removal of cyanotoxins (Nybom et al., 2007, 2008a, b). Lactic acid bacteria are natural inhabitants of the human intestinal tract and also have a long history of safe use in fermented products and other foods. Some strains of lactobacilli, bifidobacteria and also some propionibacteria have been introduced as probiotics in functional food products due to their health-promoting effects. Some specific strains of probiotic bacteria were shown to be effective in removal of different microcystins (-LR, -RR, -LF) and a combination of microcystins from the cyanobacterial extracts *Microcystis* PCC 7820 and NIES 107 (Nybom et al., 2008a). *Lactobacillus* (*L.*) *rhamnosus* strains GG and LC-705, *Bifidobacterium* (*B.*) *longum* 46, B. *lactis* 420 and Bb12 were shown to be the most effective strains in toxin removal among the 15 strains investigated.

If microcystins are degraded enzymatically in the presence of probiotic bacteria, it is likely that proteolytic enzymes are involved in this process. Proteinases of lactic acid bacteria have, in general, a cell-envelope location and could in this way hydrolyze the peptide bonds of molecules present outside the cells. On the other hand, their specificity to milk casein could mean that they are not able to decompose smaller molecules, e.g. cyclic heptapeptides. On the contrary, cytosolic peptidases have broad substrate specificities, and several different oligopeptides could be hydrolyzed by these enzymes, but their location is always intracellular (Christensen et al., 1999), requiring uptake of hypothetical substrates.

The aim of the current work was to study the removal process of microcystins by probiotic bacteria and to test the hypothesis that cell wall-associated proteinases of probiotic bacteria are involved in the process. Based on a previous report investigating the removal of cyanobacterial toxins by probiotic bacteria (Nybom et al., 2008a), the importance of glucose (as a factor activating the metabolism of probiotic bacteria) in the proteolytic activity against microcystins was assessed. The objective was to study the enzymatic nature of the investigated removal process. Furthermore, to investigate the possible pathway of toxin-degradation, the microcystin removal process by two *Sphingomonas* strains previously shown to degrade microcystins was compared with that of probiotic bacteria. The removal process and the localization of toxin during the process were also investigated using radioactively labeled microcystin.

2. Materials and methods

2.1. Chemicals

Methanol (HPLC-grade) and acetonitrile (HPLC S-grade) were from Rathburn (Walkerburn, UK). Formic acid (analytical-reagent grade) was from Riedel-de Haën (Seelze, Germany) and trifluoroacetic acid (TFA) was from Fluka (Buchs, Switzerland). Water was purified to 18.2 MΩ cm on a Milli-Q Synthesis system (Millipore, Molsheim, France). Microcystin solutions were prepared in 0.01 M PBS buffer (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2). Scintillation liquid OptiPhase HiSafe 3 was from PerkinElmer (USA).

2.2. Bacterial strains

Three probiotic bacterial strains were tested for their capacity to remove MC-LR. The commercial strains were *L. rhamnosus* GG (ATCC 53103, Valio Ltd., Helsinki, Finland), *L. rhamnosus* LC-705 (Valio Ltd) and *B. longum* 46 (DSM 14583) and they were obtained in commercial lyophilized form. The bacteria were cultured and harvested as previously reported (Nybom et al., 2007) and after washing with PBS buffer the cells were suspended in PBS with or without glucose (1 or 3%) to reach a final cell density of approximately 10^{10} colony-forming units (CFU) mL^{-1}) (described in Nybom et al., 2007).

Sphingomonas ACM 3962 (Australian Collection of Microorganisms) and *Sphingomonas* B9 (AB159609, Harada et al., 2004; Tsuji et al., 2006) were cultured in peptone-yeast extract medium and incubated for experiments for 18 h at 28 °C. The protocol for harvesting and washing the bacteria was the same as for the probiotic strains.

2.3. Microcystin

MC-LR was extracted from a culture of *Microcystis aeruginosa* PCC 7813 strain obtained from the Pasteur Culture Collection (Paris) and purified by HPLC as described earlier (Gajdek et al., 2003) and in some experiments from *Microcystis* PCC 7820 and purified by HPLC as described by Meriluoto and Spoof (2005a,b). Ten micrograms of MC-LR was dissolved in 1 ml of PBS buffer, and appropriate dilutions were made.

2.4. Enzyme assay

The standard peptidase assay was performed at 37 °C with 0.02 μmol L^{-1} Suc–Ala–Ala–Phe–AMC (Bachem, Torrance, CA) in 0.1 M Tris–HCl, pH 7.0 as a substrate and 25 μmol L^{-1} 7-amino-4-methyl-coumarin (AMC) dissolved in PBS as a standard. The density of cell suspension used in enzyme assays was approximately 10^{10} CFU mL^{-1}. Appropriate dilutions of the AMC standard (12.5 μmol L^{-1}, 6.25 μmol L^{-1} and 2.5 μmol L^{-1}) were made fresh daily. 150 μL of substrate was added to 25 μL of sample or standard ($n = 3$); 150 μL of Tris buffer was added to 25 μL of cell suspension/extract and 150 μL of substrate with 25 μL of PBS was used as the blank. After 1 h of incubation at 37 °C the reaction was stopped by cooling to 0 °C. Finally, the fluorescence of the product (free AMC) was measured in the fluorescence plate reader Victor 1420 Multilabel counter (Wallac - PerkinElmer) at excitation/emission wavelengths of 365/442 nm and the activity was expressed in micromoles of substrate hydrolyzed within 1 h.

2.5. Microcystin removal assay in the presence of viable cells

One milliliter of the bacteria suspended in PBS at a concentration of approximately 10^{10} CFU mL^{-1} with or without glucose together with 100 μg L^{-1} MC-LR (final concentration) were incubated for different times (0, 2, 4, 6, 24 h) in the same conditions as in the enzyme assay experiments ($n = 3$). After incubation, 200 μL aliquots were taken and centrifuged (12000 g, 10 min, room temperature) in 300 μL borosilicate glass tubes. The supernatant was analyzed to determine the residual MC-LR concentration by HPLC compared with a control (100 μg L^{-1} MC-LR in PBS with 1% glucose). In all experiments the MC-LR concentration was determined in the samples both immediately after dilution with cell suspension and after an appropriate time of incubation. Additionally, 100 μL of sample was used in an enzymatic assay.

2.6. Microcystin removal in the presence of bacterial cell extract

Bacterial cell extracts were prepared for *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46. 100 mL of 18 h old cultures were washed twice with PBS buffer (pH 7.0). Bacterial cell disruption was performed by ultrasonication on ice (Bandelin Sonopuls HD 2070 ultrasonic homogenizer, Bandelin Electronic, Germany), cell debris was removed by centrifugation (12000 g, 10 min, 4 °C) and the cell extract supernatant was used for the MC-LR removal assays. MC-LR (final concentration 100 μg L^{-1}) was incubated with 1.5 mL of cell extract supernatant in 1.5 mL borosilicate glass chromatographic vials under continuous reciprocal shaking (120 rotations per minute, Certomat WR, B. Braun, Melsungen, Germany). After 12, 18 and 24 h of incubation at 37 °C samples were taken, centrifuged (for removal of possible particles) and analyzed on HPLC (described in 2.11).

2.7. Extraction of MC-LR from bacterial cells and analysis of toxins using ELISA

After 24 h incubation with MC-LR at 37 °C cell suspensions of *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46 were centrifuged, the pellets were washed twice with PBS and the cells were resuspended in 75% MeOH. The cells were disrupted by ultrasonication (Bandelin Sonopuls HD 2070 ultrasonic homogenizer), centrifuged, and the residual MC-LR concentration in the supernatant was determined with an enzyme-linked immunosorbent assay (ELISA) QuantiPlate Kit for Microcystins according to the manufacturer's instructions (Envirologix, Portland, ME, USA). The limit of detection of the kit was 0.147 ppb. The amount of toxin found inside the cell pellet was compared with the amount of toxin removed during the 24 h incubation (HPLC results).

2.8. Correlation of proteolytic activity and MC-LR removal ability in the absence or presence of glucose

The bacterial cells of *L. rhamnosus* GG were suspended in PBS at a concentration of approximately 10^{10} CFU mL^{-1} together with 100 μg L^{-1} MC-LR (final concentration). Four experimental groups were performed (in triplicate): bacterial suspension with initial concentration of 1% (w/v) glucose and an extra 1% added at 3 h and 6 h – (group A); bacterial suspension in PBS at the beginning and 1% (w/v) glucose addition at 3 h and 6 h (group B); bacterial suspension in PBS and 1% (w/v) glucose addition at 6 h (group C); bacterial cells incubated without glucose (group D). After successive time of incubation, 150 μL aliquots were taken to determine the residual MC-LR concentration by HPLC as was described above and 100 μL of sample was used in an enzymatic assay.

2.9. Removal of ^{14}C-labeled MC-LR by probiotic bacteria

^{14}C-labeled MC-LR was purified from *Microcystis* PCC 7820, which was grown in 1-liter cultures in the presence of sodium ^{14}C-bicarbonate (50 mCi $mmol^{-1}$, PerkinElmer Life Sciences, USA) according to Brooks and Codd (1987) and Craig et al. (1996). The specific activity of ^{14}C-MC-LR was 13.4 μCi $mmol^{-1}$. ^{14}C-MC-LR at a concentration of 1 μg mL^{-1} was incubated with the bacterial strain *L. rhamnosus* GG. After 24 h the samples were centrifuged and the supernatant and cell pellet were separated. The pellet was dissolved in 75% MeOH, bacterial cell disruption was performed by ultrasonication on ice (Bandelin Sonopuls HD 2070 ultrasonic homogenizer) and the sample was centrifuged (12000 g, 10 min, room temperature). Both the supernatant and pelleted cell extract were analyzed for the presence of ^{14}C (1216 Rackbeta Liquid Scintillation counter, Wallac, Finland). Simultaneously, the removal of MC-LR was monitored on HPLC and fractions were collected for determination of ^{14}C-MC-LR associated with the cells or present in the supernatant.

2.10. Comparison of MC-LR removal and activity of cell wall-associated proteinases in the presence of proteinase inhibitors

To check the type of proteinase, an 18 h old culture of *L. rhamnosus* GG washed with PBS buffer (10^{10} CFU mL^{-1}) was incubated for 0.5 h with different doses of typical inhibitors of metalloprotease (EDTA; 1, 5 and 10 mmol L^{-1}), serine protease (phenylmethylsulfonyl fluoride (PMSF) diluted in methanol; 0.5, 5 and 50 mmol L^{-1}) and cysteine protease (iodoacetic acid; 0.01, 0.05, 0.1, 0.5 and 1 mmol L^{-1}) (all obtained from Sigma). Additionally, the effect of a protease inhibitor cocktail (Sigma) containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, and sodium EDTA with final concentrations of 2.32, 0.03, 0.2, 0.033 and 10 mmol L^{-1}, respectively, was studied. To exclude the impact of the inhibitors on the viability of the bacterial cells, traditional plate counting was performed at the beginning of the experiment and after 6 h of incubation using MRS agar plates. Based on the results of this experiment, the involvement of EDTA in MC-LR removal was investigated in detail. Using the same bacterial culture, a part of the sample was taken after 0, 2, 4 and 6 h to measure the proteolytic activity and the concentration of microcystin; assays were performed as described in 2.4.

2.11. HPLC analysis

MC-LR was quantified by HPLC on a Purospher STAR RP-18 endcapped column (55 mm × 4 mm, 3 μm particles; Merck, Darmstadt, Germany) as described by Meriluoto and Spoof (2005a) and the analysis was performed as described by Nybom et al. (2007). For experiments with ^{14}C-MC-LR and MC-LR removal by *Sphingomonas* strains, a Purospher STAR RP-18 endcapped column (250 mm × 4 mm, 5 μm particles; Merck, Darmstadt, Germany) was used. The mobile phase consisted of a gradient of 0.05% aqueous trifluoroacetic acid (solvent A) and 0.05% TFA in acetonitrile (solvent B) with a linear gradient program (5–70% B over 40 min), injection interval 60 min.

2.12. LC-MS analysis

The instrument consisted of an Agilent Technologies (Waldbronn, Germany) 1200 Rapid Resolution LC coupled to a Bruker Daltonics HCT Ultra Ion Trap MS with an electrospray ion source. The column, a Purospher STAR RP-18 endcapped column (55 mm × 4 mm, 3 μm particles; Merck), was kept at 40 °C, the injection volume was 5 μL. The ion trap was operated in the standard enhanced scan mode from *m/z* 300 to 1200. The ICC target was set to 300,000 with a maximum accumulation time of 100 ms. The ion source parameters were: dry temperature 365 °C, nebulizer pressure 60 psi and dry gas flow 12 L min^{-1}. The mobile phase consisted of a gradient of 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) with the following linear gradient program: 25% B to 70% B over 5 min, then to 90% B over 2 min, where it was held for 1 min. The injection interval was 10 min. Data analysis was performed with the Bruker Compass software version 1.1.

2.13. Toxicity assay

Toxicity of the MC-LR samples after incubation with cells of *L. rhamnosus* GG and *L. rhamnosus* LC-705 was established by protein phosphatase 1 (PP1) inhibition assay as described by Meriluoto and Spoof (2005b). The concentration of MC-LR (standard) for preparing the calibration curve ranged between 0.125 and 4.0 μg L^{-1}. Part of the samples, which were analyzed by HPLC and in enzymatic assays, were frozen and diluted directly before the experiment to obtain an MC-LR concentration between 0.5 and 2.0 μg L^{-1}. The toxicity values were converted into MC-LR equivalents.

2.14. Statistics

Statistical analysis was performed using the *t* test (Microsoft Excel software). Pearson test was used to indicate the correlation between the proteinase activity and the MC-LR removal.

3. Results

3.1. Removal of MC-LR by whole cells versus bacterial cell extracts

Fig. 1 shows the comparison of MC-LR removal by cell solutions of *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46 and the cell extracts of the same strains. Incubation with bacterial cell solutions (10^{10} CFU mL^{-1}) resulted in a 55–70% reduction of MC-LR within 24 h of incubation at 37 °C. In the presence of bacterial cell extracts MC-LR was not removed from solution by any of the three studied probiotic strains (less than 5% removal). These findings indicate that activity related to intact bacterial cells is needed for efficient microcystin removal.

3.2. Analysis of MC-LR extracted from cell pellets using ELISA and comparison with HPLC

Bacterial cells incubated at 37 °C for 24 h with MC-LR were disrupted and the toxin associated with the cell wall, membranes and cytosolic fraction of cells was analyzed using ELISA. As the HPLC results show, around 55–70% MC-LR was removed by *L. rhamnosus* GG, LC-705 and *B. longum* 46 within 24 h, but only a small fraction of the removed toxin (less than 8%) could be found accumulated by or associated to the bacterial cells (Table 1).

3.3. Radioactivity assay: removal of ^{14}C-labeled MC-LR and localization of radioactivity

Experiments with radiolabeled microcystin were performed to investigate whether microcystin is transported into the bacterial cells during toxin removal. The experiments were performed using ^{14}C-MC-LR (final concentration 1 μg mL^{-1}). The analysis of ^{14}C-location indicated that after 0, 6, 12, 18 and 24 h of incubation of radiolabeled MC-LR with *L. rhamnosus* GG, the radioactivity was found only

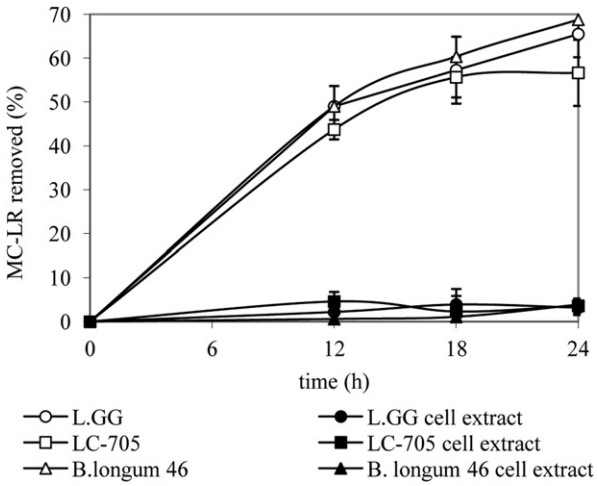

Fig. 1. Removal of MC-LR by probiotic bacterial cells versus cell extracts (*L. rhamnosus* GG, *L. rhamnosus* LC-705, *B. longum* 46). Initial MC-LR concentration 100 μg L^{-1}, bacterial concentration 10^{10} CFU mL^{-1}, temperature 37 °C; average ± SD, $n = 3$.

Table 1
ELISA-Analysis of MC-LR extracted from cell pellets and comparison with HPLC.

Bacterial strain	MC-LR removed in % (HPLC)	MC-LR removed in ng (calculated)	MC-LR in cell pellet in ng (ELISA)
L. rhamnosus GG	63.5 ± 4.2	95.3	7.3 ± 2.9
L. rhamnosus LC-705	57.7 ± 5.9	86.6	6.2 ± 3.6
B. longum 46	68.7 ± 4.8	103.1	6.9 ± 1.8

Bacterial concentration 10^{10} CFU mL^{-1}, 24 h incubation at 37 °C with MC-LR (100 μg L^{-1}).

in the supernatant (Table 2). Only a small fraction of toxin (less than 3% of radioactivity) was found in the cell pellets of disrupted bacterial cells during the process of MC-LR elimination, which excludes toxin degradation inside the cells by cytosolic proteases and the contribution of cell-associated proteinases. These findings suggest that microcystin is not transported through the membrane of the bacterial cells and does not associate with the cell-wall or proteins of the cell membrane, and that the removal observed is due to a process that occurs outside of the cells.

3.4. Activity of cell-envelope proteinases and MC-LR removal

No proteolytic activity against Suc–Ala–Ala–Phe–AMC was found in the supernatant obtained by centrifugation of the bacterial cell cultures of the investigated strains *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46. Enzymatic activity was found only in the cell suspensions and was much higher when measured in the cell suspension incubated with 1 or 3% glucose than after incubation with PBS (Fig. 2). The activity decreased during incubation and had disappeared almost completely after 24 h of incubation.

Simultaneous analysis of the enzymatic activity and the rate of MC-LR removal indicated that these parameters are very closely related to each other and both decreased with time (Fig. 3). In particular, the enzymatic activity and the concentration of MC-LR changed radically within the first 6 h of incubation. Significant correlation between enzymatic activity and the removal of MC-LR was found for three investigated strains ($r = 0.97$, $F = 126.5$, $P < 0.01$ for *L. rhamnosus* LC-705; $r = 0.88$, $F = 27.18$, $P < 0.01$ for *L. rhamnosus* GG and $r = 0.95$, $F = 29.5$, $P < 0.01$ for *B. longum* 46). The close dependence of the proteolytic activity and the removal in presence of glucose as an additional energy source were indicated in the correlation experiment (data summarized in Table 3). In group A (increased concentration of glucose from 1% at 0 h to 3% at 6 h) the percentage of removed MC-LR was highest – 77.9 and 76.9%, respectively. For 3 experimental groups (A–C) the highest proteolytic activity was observed directly after the first doses of glucose. Similarly, MC-LR was removed with the fastest rate during the three first hours following glucose addition. After the next dose of glucose (group A and B) the enzymatic activity and the rate of toxin removal was lower but still significantly higher than before glucose addition.

3.5. Experiments with protease inhibitors

The activity of cell-associated proteinases of *L. rhamnosus* GG was measured after incubation with different concentrations of protease inhibitors (Table 4). For EDTA the concentration of inhibitor causing considerable reduction of measured activity was 10 mmol L^{-1} (48% of activity in comparison with control). Furthermore, proteinases were inhibited at a similar level (60% of activity in comparison with control) in the presence of a protease inhibitor cocktail containing 10 mmol L^{-1} of EDTA (Table 4 and Fig. 4a). The results suggest that the main proteolytic activity observed for this strain is due to metallo-enzymes. PMSF did not change the activity of cell-envelope proteinases even at a high concentration (50 mmol L^{-1}). Additionally, almost complete abolition of measured proteolytic activity was observed after incubation with 0.1 mmol L^{-1} iodoacetic acid (15% of activity in comparison with control).

The viability of *L. rhamnosus* GG cells was not changed after incubation with 10 mmol L^{-1} EDTA, whereas significant decrease of viable cells was observed after incubation with 0.1 mmol L^{-1} iodoacetic acid (Table 4). Within 6 h of incubation with this inhibitor the cell density measured using MRS agar plates decreased from 10^{10} to below 10^5 CFU mL^{-1}, on average.

Because EDTA was chosen as a potent proteinase inhibitor, a detailed analysis of its impact on the proteolytic activity and removal ability was performed. It was found that the rate of MC-LR removal was significantly slower and after 6 h of incubation with bacterial cells only 19% and 10% of MC-LR was removed in the presence of EDTA and the protease inhibitor cocktail, respectively (Fig. 4b). A correlation between proteinase activity and MC-LR removal was also indicated when these parameters were measured simultaneously (Fig. 5).

3.6. Comparison of MC-LR degradation products for *Sphingomonas* strains and probiotic bacteria

The removal process of MC-LR by the probiotic strain *L. rhamnosus* GG was compared with two *Sphingomonas* strains, ACM 3962 and B9, and the degradation products

Table 2
Location of ^{14}C from radiolabeled MC-LR after incubation with *L. rhamnosus* GG found in the supernatant (0, 6, 12, 18 and 24 h) and cell pellet (24 h).

Time (h)	0	6	12	18	24	Cell pellet
Amount of ^{14}C (cpm)	581 ± 42	596 ± 43	634 ± 34	590 ± 46	572 ± 34	14 ± 3.8
% of radioactivity	100	102.6	109.1	101.5	98.5	2.8

^{14}C-MC-LR concentration 1 μg mL^{-1}, radioactivity found in PBS buffer subtracted from results (control sample).

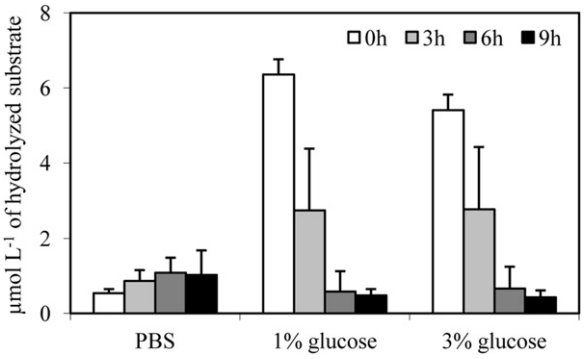

Fig. 2. Proteolytic activity of cell suspensions of *L. rhamnosus* GG measured at 0 h and after 3, 6 and 9 h of incubation with PBS, 1% glucose and 3% glucose. Bacterial concentration 10^{10} CFU mL^{-1}; average ± SD, $n = 3$ (the data for *L. rhamnosus* LC-705 and *B. longum* 46 were analogous with the presented one).

were identified. Fig. 6a shows the HPLC chromatograms for the strains. A reduction of MC-LR by the probiotic strain and the two *Sphingomonas* strains was shown. A maximum removal of around 70–80% of MC-LR was observed by *L. rhamnosus* GG after 24 h of incubation, and the removal efficiency of the *Sphingomonas* strains was similar to that of the probiotic bacteria. In addition to reduction of the MC-LR peak, additional peaks were observed for ACM 3962 and B9 after incubation, while for the probiotic bacteria no new peaks were observed. MC-LR eluted at 18.1 min, linear MC-LR at 11.5 min and the tetrapeptide degradation product at 13.9 min (Fig. 6a).

The degradation products of MC-LR produced by the *Sphingomonas* strains were confirmed to be the same as previously reported; linearized MC-LR (Adda–Glu–Mdha–Ala–Leu–Masp–Arg–OH) and a tetrapeptide (Adda–Glu–Mdha–Ala–OH). The linear MC-LR and the tetrapeptide were seen in the HPLC chromatogram of the ACM strain, but for B9 the linearized MC-LR had already been further degraded and only the tetrapeptide was seen (Fig. 6a). The products and degradation patterns (not shown) were identified by HCT Ultra Ion Trap LC-MS (Fig. 6b and Table 5). Linearized MC-LR (major peak *m/z* 862) and the tetrapeptide (*m/z* 615) were observed for the two *Sphingomonas* strains. These degradation products were not obtained using the probiotic strains, which suggests that the removal mechanisms are not the same. Furthermore, no additional degradation products could be observed or identified from samples incubated with the probiotic strain.

3.7. PP1 inhibition assay

Additionally to the experiments described above, the toxicity assay was performed for selected samples. In comparison with control (MC-LR before incubation with bacterial cells) the toxicity decreased for *L. rhamnosus* LC-705 to 60.0% (6 h) and 28.1% (24 h); for *L. rhamnosus* GG to 49.0% (6 h) and 33.3% (24 h). The calculated concentration of MC-LR decreased simultaneously with the values obtained during removal experiments, measured by the HPLC method (Table 6).

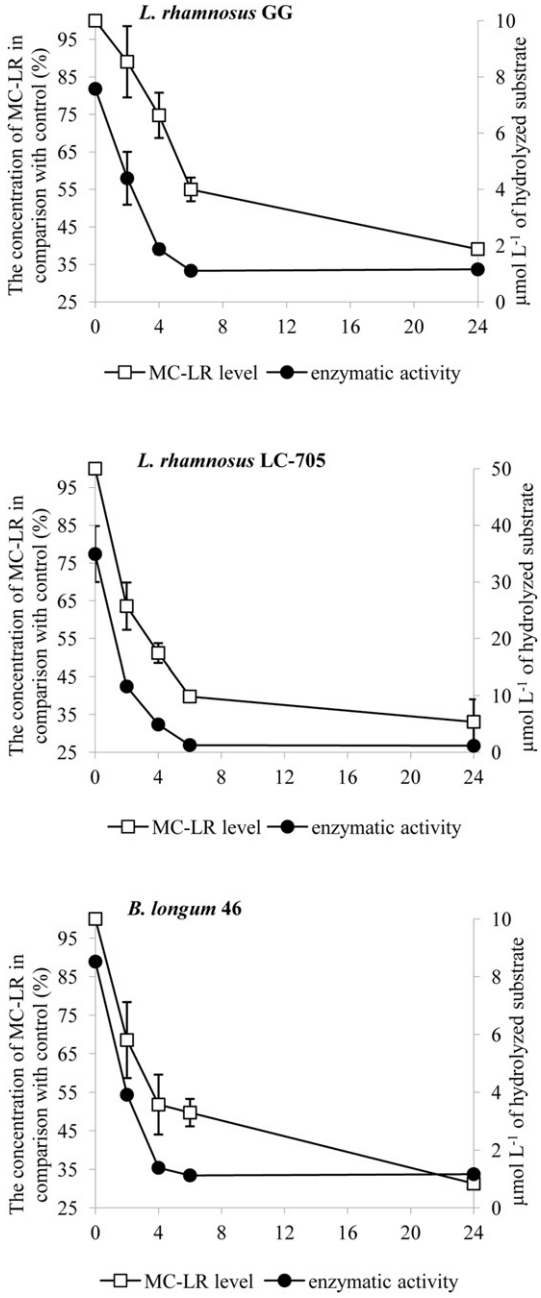

Fig. 3. The correlation of changes in MC-LR removal and proteinase activity measured within 24 h of incubation for three probiotic strains. Initial MC-LR concentration 100 μg L^{-1}, bacterial concentration 10^{10} CFU mL^{-1}, temperature 37 °C, average ± SD, $n = 3$.

4. Discussion

Several authors have described the biodegradation of hepatotoxins in the presence of naturally occurring bacterial strains (Cousins et al., 1996; Park et al., 2001; Harada et al., 2004; Kato et al., 2007). An enzymatic pathway for the bacterial degradation of toxic cyclic peptides mediated by proteolytic enzymes of *Sphingomonas* species was postulated by Bourne et al. (1996). These proteins (MlrA,

Table 3
The rate of MC-LR removal and the proteolytic activity of cell-envelope proteinases against Suc–Ala–Ala–Phe–AMC for *L. rhamnosus* GG.

Incubation time (h)	The percentage of MC-LR (initial concentration 100 μg L^{-1}) removed within 1 h and μmol L^{-1} of hydrolyzed substrate per 1 h (*italized*) for four experimental groups							
	A		B		C		D	
0		***6.08***		*0.68*		*0.40*		*0.48*
3	**11.15**	***3.56***	**1.83**	***6.92***	2.51	*0.81*	1.52	*0.82*
6	**6.78**	***0.79***	**9.02**	***4.05***	**1.79**	***6.57***	2.66	*0.99*
9	3.42	*0.36*	3.94	*0.71*	4.56	*2.65*	0.99	*1.14*
24	1.08	*0.42*	1.20	*0.44*	1.68	*0.53*	0.32	*1.09*
% of MC-LR removed after 24 h	77.89[a]		72.23		62.47		20.35	

Bold values indicate the time of glucose addition.
[a] statistically significant differences in comparison with cells incubated with 1% glucose (64.39% of removed MC-LR within 24 h).

MlrB and MlrC) and their encoding genes involved in the bacterial hydrolysis of microcystins were also characterized (Bourne et al., 2001; Saito et al., 2003). Recently, the removal of different microcystins by strains of metabolically active probiotic bacteria was documented. As shown by Nybom et al. (2008b) high MC-LR removal was observed with the probiotic bacterial strains *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46. Freshly cultured bacteria were shown to be more efficient in microcystin-removal than lyophilized or non-viable bacteria (Nybom et al., 2007, 2008a). The removal efficiency was also dependent on temperature, pH and cell density, and increased significantly when glucose was added to the medium (Nybom et al., 2008a). A maximum removal of 95% was observed for *Lactobacillus plantarum* strain IS-20506 (37 °C, 10^{11} CFU mL^{-1}) with 1–2% glucose supplementation (Nybom et al., 2008a).

4.1. No activity against MC-LR present in cytosolic fraction of probiotic bacteria

The importance of cell viability as well as improvement of microcystin removal after addition of glucose suggested that a biological process rather than physical absorption to the cell wall is responsible for microcystin removal by probiotic bacteria. The proteolytic system present in all species of lactic acid bacteria was the natural candidate for this study. It is composed of 1) extracellularly located proteinases involved in the initial cleavage of casein to peptides, 2) peptidases that hydrolyze the large peptides thus formed into smaller peptides and 3) amino-acids and peptide transport systems involved in the cellular uptake of the hydrolytic products (Law and Haandrikman, 1997).

Based on the experiments we conclude that no activity against MC-LR is present in the cytosolic fraction of the investigated probiotic strains (Fig. 1), which excludes the involvement of intracellular proteases in the process of MC-LR removal. Additionally, the studies with ^{14}C-radio-labeled microcystins confirm that MC-LR is not transported into the cells of probiotic bacteria during incubation with bacterial cells, (Table 2). ELISA analysis of toxin found inside bacterial cells after incubation with MC-LR showed supporting results (Table 1). Furthermore, bacterial cell extracts were not able to remove MC-LR (Fig. 1). It was concluded that the peptide transport system is not involved in the process of microcystin-removal and degradation inside the cell by cytosolic proteases is not present in the probiotic bacteria.

4.2. Enzymatic activity and rate of MC-LR removal are related

Consequently, the participation of cell-envelope proteinases in the removal of MC-LR was investigated. The biochemistry of these enzymes in lactococcal strains is well known. Solubilized lactococcal proteinases have high molecular masses, a pH optimum between 6 and 7, and are strongly inhibited most often by metal-chelating compounds (EDTA) or phenylmethanesulfonyl fluoride (PMSF), suggesting they are serine-type proteinases. The proteinases present in lactobacilli are very similar to the

Table 4
Proteolytic activity of cell wall-associated proteinases and the viability of *L. rhamnosus* GG cells after 6 h of incubation at 37 °C with EDTA, PMSF, iodoacetic acid and protease inhibitor cocktail.

Type of inhibitor	Control (PBS + 1% glucose)[a]		Cells incubated with inhibitor	
	Proteolytic activity (μmol L^{-1} of hydrolyzed substrate per 1 h)	Cell counts (CFU mL^{-1})	Proteolytic activity (μmol L^{-1} of hydrolyzed substrate per 1 h)	Cell counts (CFU mL^{-1})
EDTA (10 mmol L^{-1})	7.57 ± 0.88	3.0×10^{10}	3.66 ± 0.78	2.1×10^{10}
PMSF (50 mmol L^{-1})	7.57 ± 0.88	2.2×10^{10}	7.24 ± 1.22	–
Iodoacetic acid (0.1 mmol L^{-1})	7.57 ± 0.88	2.8×10^{10}	1.14 ± 0.43	Below 10^5
Protease inhibitor cocktail[b]	6.87 ± 0.39	–	4.09 ± 0.14	–

Only the values for concentrations causing significant reduction of proteolytic activity are shown; for PMSF the value for highest concentration used is presented.
[a] for protease inhibitor cocktail control samples were incubated with DMSO 5 times diluted with water.
[b] final concentrations of inhibitors were as follows: 2.32 μmol L^{-1} AEBSF, 0.03 μmol L^{-1} pepstatin A, 0.2 μmol L^{-1} E-64, 0.033 μmol L^{-1} bestatin, and 10 μmol L^{-1} sodium EDTA.

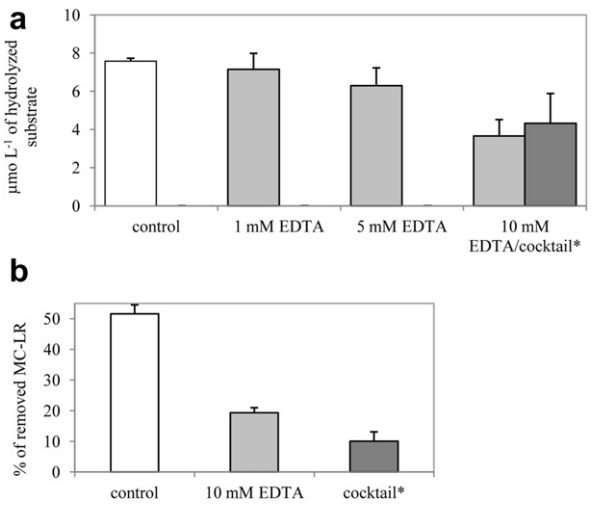

Fig. 4. The proteolytic activity of cell wall-associated proteinases of *L. rhamnosus* GG after (a) 0.5 h of incubation with different doses of EDTA and (b) the percentage of removed MC-LR after 6 h incubation with 10 mmol L^{-1} EDTA; average ± SD, $n = 3$. Asterisk indicates the values of measured parameters in the presence of a protease inhibitor cocktail with 10 mmol L^{-1} of EDTA.

lactococcal proteinase (Law and Haandrikman, 1997). We found that proteinase activity is present only in the cell suspension and no activity is expressed in the supernatant, which indicates that active enzymes are not released from

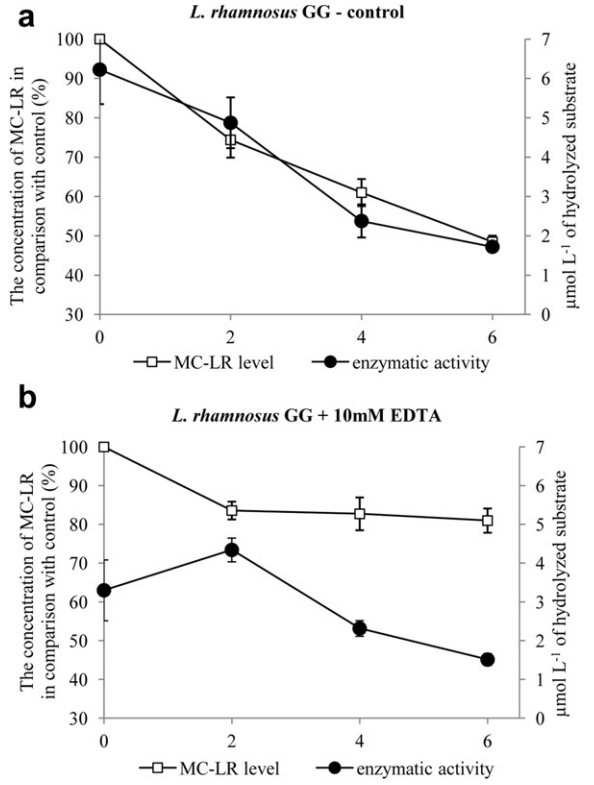

Fig. 5. MC-LR level and proteinase activity of *L. rhamnosus* GG incubated in (a) standard condition and (b) with 10 mmol L^{-1} EDTA; average ± SD, $n = 3$.

the cells. A large increase of enzymatic activity was observed after addition of 1 or 3% glucose (Fig. 2). Furthermore, after 6 h of incubation a significant reduction of Suc–Ala–Ala–Phe–AMC hydrolysis was shown. These findings suggest that an energy source is required to maintain high proteolytic activity and, in addition, glucose consumption may be responsible for the decrease of this activity.

Simultaneous analysis of the enzymatic activity and the rate of MC-LR removal indicated that these parameters are very closely related to each other and both decreased with time (Fig. 3 and Table 3). Despite similar microcystin removal ability the proteinase activity differed significantly among the strains used, which could be explained by various proteinase specificities towards microcystin as a substrate. In particular, the enzymatic activity and the concentration of MC-LR changed radically within the first 6 h of incubation. It must be stressed that both parameters increased when glucose was added. The next dose of glucose retained relatively high enzymatic activity and toxin removal efficiency. The highest values of measured parameters were indicated for groups with 3% glucose as a final concentration (A). Glucose available for the cells was important in retaining high metabolic activity for several hours.

4.3. Removal of MC-LR decreases in the presence of metallo-peptidase inhibitor

Experiments with inhibitors (Table 4) allowed concluding that metallo-peptidases are the main enzymes anchored to the cell wall. This finding is in agreement with other studies, as extracellular proteinases identified in lactic acid bacteria are generally metallo-enzymes or serine proteases (Stefanitsi et al., 1995; Guo et al., 2009). A detailed analysis of the impact of inhibitors on the proteolytic activity and toxin removal ability indicated that for the investigated strain (*L. rhamnosus* GG) the removal rate of MC-LR was approximately 3 and 4 times slower when cells were incubated with EDTA and protease inhibitor cocktail, respectively (Fig. 3b). This finding further confirmed that extracellular proteinases are involved in the process of microcystin-degradation. The addition of 10 mmol L^{-1} EDTA inhibited MC-LR removal (Fig. 5), which was much slower during 6 h of the experiment. All these results are in agreement with previous findings that EDTA affects the proteinase activity of probiotic bacteria and that this activity correlates with the process of MC-LR removal.

4.4. Microcystin-removal in the presence of probiotic bacteria differs from biodegradation by Sphingomonas

During the last few years PCR assays have been used by several authors (Bourne et al., 2005; Hoefel et al., 2009) to screen the presence of genes (primary found in *Sphingomonas* sp.) involved in microcystin-degradation, but only among strains occurring in natural water environments. However, screening of the known sequences deposited in GenBank did not indicate the presence of any homologues in the genome of lactic acid bacterial strains. It does not exclude the ability of probiotic bacteria to degrade

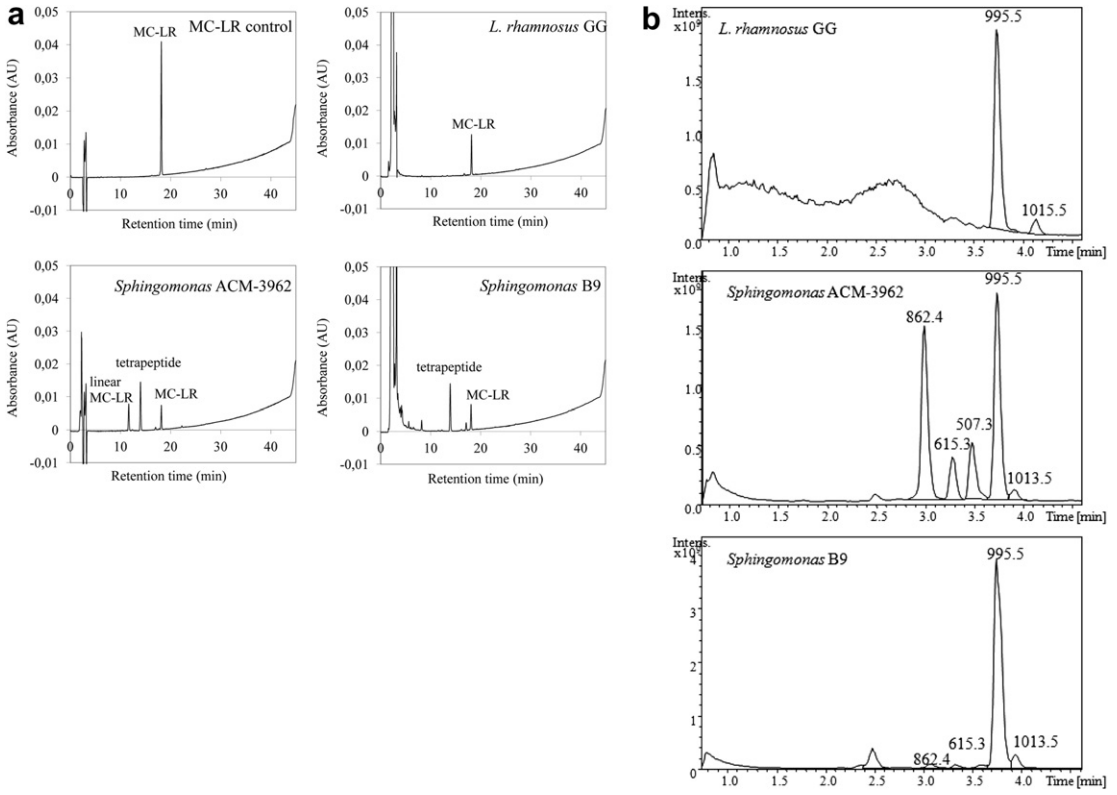

Fig. 6. Comparison of MC-LR removal by *L. rhamnosus* GG, *Sphingomonas* ACM 3962 and B9. (a) HPLC chromatograms of bacteria incubated with MC-LR for 24 h at 28 °C (ACM and B9) or 37 °C (*L. rhamnosus* GG), (b) LC-MS chromatograms of the same samples, showing degradation products (listed in Table 5).

microcystins, but suggests different ways of such degradation. Furthermore, our findings demonstrate that the process of microcystin-removal in the presence of probiotic bacteria differs from known mechanisms of biodegradation that occurs in the cells of *Sphingomonas* (Fig. 6). Previous studies have shown that strains of *Sphingomonas* are able to degrade microcystins by intracellular hydrolytic enzymes (Bourne et al., 1996; Harada et al., 2004; Ishii et al., 2004) and the key enzyme involved in microcystin-degradation, MlrA, is present in the periplasmic space and digested peptides are transported into the cell by specific transporters (Bourne et al., 2001; Ishii et al., 2004). On the contrary, in the case of probiotic bacteria the enzymatic degradation seems to occur outside the cells. Unfortunately, LC-MS analyses did not provide any data about products of such degradation for the studied probiotic strain and this issue is therefore the aim of future investigations. The PP1 inhibition assay performed for selected samples indicated that MC-LR removal results in parallel reduction of toxicity (Table 6). This is extremely crucial in the context of possible exploitation of lactic acid bacterial strains against this kind of common contamination.

Table 5
Degradation products found for *Sphingomonas* strains.

Toxin or degradation product and fragment ion	*m/z*
MC-LR $[M + H]^+$	995.5
Adda–Glu–Mdha–Ala–OH $[M + H]^+$	615
Adda–Glu–Mdha–Ala–Leu–Masp –Arg–OH $[M + H]^+$	1013.5
Adda–Glu–Mdha–Ala–Leu–Masp –Arg–OH $[M + 2H]^{2+}$	507
Adda–Glu–Mdha–Ala–Leu–Masp –Arg–OH — NH2 — PhCH2CHOMe	862

HCT Ultra Ion Trap LC-MS analysis.

In summary, our experiments have for the first time provided evidence that cell wall-associated proteinases of specific probiotic strains could be involved in the process of microcystin-removal. Strong correlation between the activity of cell-envelope proteinases and the decrease of MC-LR concentration suggests that these enzymes are involved in microcystin removal. Furthermore, experiments with inhibitors documented that EDTA, which was found as a main inhibitor of proteinases of the investigated strain *L. rhamnosus* GG, was shown to reduce the rate of microcystin-removal, which is in agreement with previous conclusions. PP1 inhibition assay confirmed the efficiency of microcystin-detoxification of the strains studied. All these findings strongly support the hypothesis that enzymatic degradation of microcystin occurs when the toxin is incubated with cell suspensions of probiotic bacteria. The characterization of the investigated process sheds new light on the ways of cyanotoxin removal and could be useful in future exploitation of probiotic strains. The major route to microcystin-exposure is through water intake and intestinal absorption (Ito et al., 2000). The effect of

Table 6
Comparison of MC-LR removal process measured by HPLC and the toxicity of the samples measured by PP1 inhibition assay.

Strain	Percentage of removed MC-LR measured by:	
	HPLC method	PP1 inhibition assay
L. rhamnosus LC – 705 – 6 h	43.4 ± 7.6	39.9 ± 25.7
L. rhamnosus LC – 705 – 24 h	71.6 ± 9.6	71.7 ± 7.6
L. rhamnosus GG – 6 h	47.4 ± 9.5	51.0 ± 16.7
L. rhamnosus GG – 24 h	64.0 ± 1.0	66.5 ± 15.0

Toxicity values obtained by PP1 inhibition assay were converted to MC-LR concentrations.

exposure to sub-lethal levels of microcystins is usually even not noticed because of the lack of well-defined clinical symptoms. Further research has been suggested to find possible ways of treatment for humans exposed to the toxin. Probiotic bacteria constitute promising candidates that could be used both as degraders of cyanotoxins present in drinking water and for a personal gastro-intestinal protection against contaminated drinking water and diet. Thus, understanding the mechanism of microcystin removal is crucial in efficient employment of bacterial cells or their enzymes. Future experiments should focus on the isolation and purification of cell-envelope proteinases and testing of the degradation of microcystin in the presence of purified enzymes.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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