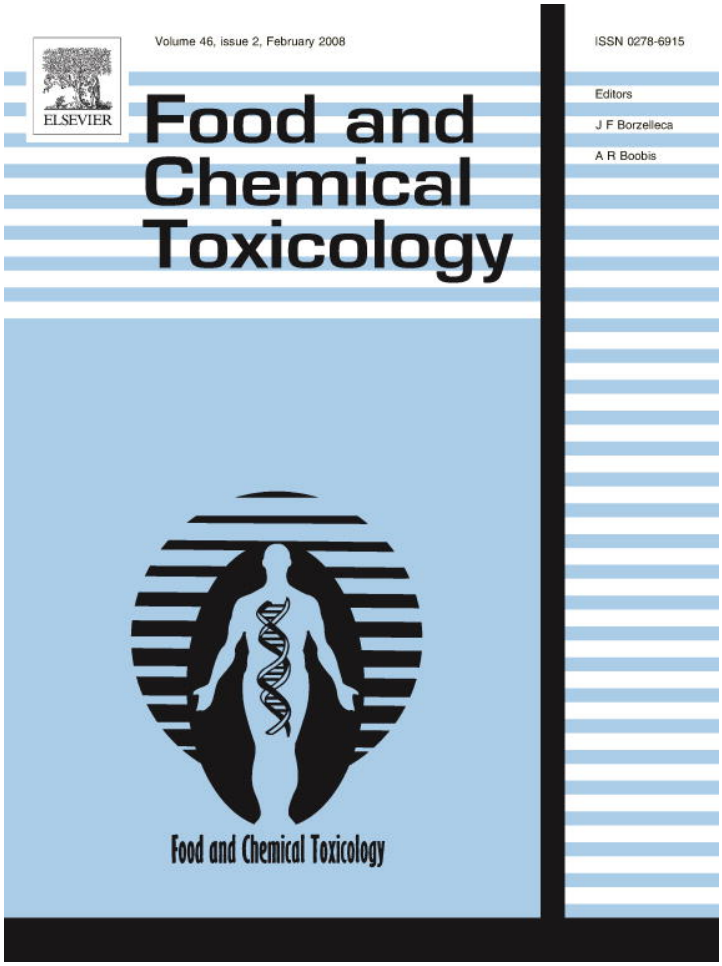

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Effect of glucose and incubation temperature on metabolically active *Lactobacillus plantarum* from dadih in removing microcystin-LR

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Abstract

Lactobacillus plantarum strains IS-10506 and IS-20506 isolated from Indonesian traditional fermented milk, dadih, were screened for their ability to remove the cyanobacterial toxin microcystin-LR (MC-LR) from aqueous solution (100 μg/L) at 22 and 37 °C. The objective was to study the main environmental factors influencing the metabolic activity of *L. plantarum* in MC-LR removal. Residual MC-LR was quantified using HPLC. Non-viable cells inactivated by boiling or acid showed only low MC-LR removal (<23 %). Viable *L. plantarum* strain IS-10506 at pH 7, at 22 and 37 °C was able to remove MC-LR, 64% and 43%, respectively, after 30 h. Strain IS-20506 at pH 7, at 22 and 37 °C removed 92% and 45 %, respectively, after 30 h. At 37 °C, the removal of MC-LR was lower than at 22 °C. Supplementation with glucose (1%, 2%, and 3%, w/v) resulted in faster and higher removal of MC-LR at 37 °C, while at 22 °C it did not improve MC-LR removal. In the presence of 1 % glucose, IS-10506 and IS-20506 demonstrated significantly the most efficient removal of 80% and 65% of applied MC-LR, after 25 and 20 h, respectively, at pH 7, 37 °C. Viable cells as well as active metabolism play important roles in removing MC-LR. This finding offers new and economical tools for decontaminating microcystin containing water.

Keywords: Microcystin-LR; Temperature; Glucose; Dadih; *Lactobacillus plantarum*

1. Introduction

Microcystins are the main toxins produced by cyanobacteria. They are cyclic peptides classified as hepatotoxins and tumor promoters. A provisional guideline level with a limit of 1 μg of microcystin-LR (MC-LR) per liter in drinking water has been established for the protection of human health protection by the World Health Organization (WHO, 1998). Microcystins are chemically stable compounds (Harada, 1996; Lahti et al., 1997). Conventional drinking water treatment has only limited efficacy in removing dissolved MC-LR (Svrcek and Smith, 2004).

Dadih, a yogurt-like product, is an Indonesian traditional fermented product from West Sumatra, which is spontaneously fermented from fresh raw buffalo milk in bamboo tubes capped with banana leaves (Akuzawa and Surono, 2002). The raw buffalo milk, the inner part of bamboo tubes, or the banana leaves are the most probable entry routes for the indigenous lactic acid bacteria *L. plantarum* strains found in dadih. Dadih has a long history of safe use as food in West Sumatra. Moreover, a recent post-market surveillance study showed that *L. plantarum* was not found in bacteremia cases (Salminen et al., 2002). *L. plantarum* is a widely distributed species in most fermented products of animal or plant origin, where it is either used in

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controlled fermentation or derived from the environment and emerges after manufacture (Corsetti and Gobbetti, 2002). It is also accepted in the European Food Safety Authority Qualified Presumption of Safety of Microorganisms in Food and Feed (Anonymous, 2005). Thus, *L plantarum* strains are safe and can be added to food and drinks, facilitating also their safe application to water.

Our work was designed to study the main environmental factors influencing the metabolic activity of food grade *L. plantarum* strains from dadih in removing MC-LR. The overall aim was to assess the optimum temperature and role of glucose in activating the metabolism of *L. plantarum* from tropical food origin. The possible microcystin removal capacity may be used for water decontamination purposes, and to promote human health in rural areas.

2. Materials and methods

2.1. Bacterial cultures

The two dadih lactic acid bacteria strains used in this study, identified by the partial sequence of 16S ribosomal RNA gene as *Lactobacillus plantarum,* have respective identification numbers IS-10506 (Gene Bank Accession number DQ860148) and IS-20506 (Gene Bank Accession number DC860149) at the University of Turku culture collection. The lactic acid bacteria were precultured in the Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK), at 37 °C, harvested after 15 h incubation by centrifugation (3220*g,* 4 °C, 20 min), washed three times with PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.0), and used for MC-LR removal experiments. The purity of freshly grown cells were tested before each experiment, and viability was analysed after defined incubation times with microcystin-LR.

Inactivation of both strains with heat treatment for 30 min at 100 °C or with 1 mol/L HCl was carried out to prepare non-viable cells.

2.2. Viability assay

2.2.1. Total viable counts by plate counting

A total viable count (cfu/mL) was measured by appropriate decimal dilutions and plate counting on MRS agar plates. Two plates per treatment were incubated at 37 °C for 48 h in aerobic conditions.

2.2.2. Viable counts by fluorescent counts

Counts of total, viable and dead bacterial cells (cells/mL) were obtained by the use of LIVE/DEAD BacLight Bacterial Viability Stain Kit (Molecular Probes). This viability kit distinguishes live bacterial cells from dead and damaged cells by means of membrane integrity. This kit combines the membrane-permeant green fluorescent nucleic acid dye SYTO 9 and the membrane-impermeant red fluorescent nucleic acid dye propidium iodide (PI), staining membrane of damaged cells fluorescent red and intact cells fluorescent green.

Bacterial cells were washed twice with PBS buffer (pH 7.2) after overnight growth. Ten microliters of cell suspension was mixed with 1.5 μL of a mixture of SYTO9 and propidium iodide (1:1), nucleic acid stains from the LIVE/DEAD BacLight kit, vortexed and incubated in the dark for 15 min at room temperature (22 °C) according to manufacturer's instruction. Immediately prior to analysis, 10 μL of fluorospheres (Molecular Probes) was added to each sample to obtain the absolute bacterial cell counts by flow cytometry (BD FACSCalibur, Becton Dickinson, USA).

2.3. Cyanotoxin preparation

Microcystin-LR (MC-LR) was purified according to Spoof and Meriluoto (2005). Ten micrograms of MC-LR was dissolved in 1 mL of ultrapure water, diluted and used for the experiments. All other reagents and solvents were either of analytical reagent or chromatographic grade.

2.4. Microcystin-LR removal assay

A specific concentration of freshly grown lactic acid bacteria cells (10^{10} cfu/mL) was suspended in PBS (pH 5.0 or 7.0). The suspended bacteria together with MC-LR was incubated in 1.5 mL borosilicate glass chromatographic vials under continuous reciprocal shaking (Certomat WR, B. Braun, Melsungen, Germany; 120 rotations per min) at 22 °C and 37 °C for defined incubation times (15, 20, 25 and 30 h), and centrifuged (12,000*g*, 22 °C, 8 min) in 200 μL borosilicate glass tubes. The supernatants were analysed to determine the residual MC-LR concentration by high-performance liquid chromatography (HPLC) according to the method described earlier (Meriluoto et al., 2005). The detection limit for MC-LR was 0.8 ng per 30 μL injection ($S/N = 3$).

A specific concentration of lactic bacteria cells (10^{10}–10^{11} cfu/mL) was also suspended in 1.5 mL PBS (pH 7.0) with 0%, 1%, 2% or 3 % glucose (w/v). To these suspensions, MC-LR solution was added, giving a final concentration of 100 μg/L.

The removal percentage was calculated as (peak area of MC-LR solution with the lactic bacteria cells at 0 h minus peak area of the MC-LR solution with the lactic bacteria cells at defined time)/(peak area of MC-LR solution with lactic bacteria cells at 0 h) × 100%. Experiments were carried out in triplicate.

2.5. Statistics

Analysis of variance (ANOVA) was done using the software Statistical Analysis Systems, SAS version 9.1 for Windows (SAS Institute Inc., Cary, NC, USA). Mean comparisons were performed using the Tukey studentized range test. The probability level of 5% ($\alpha = 0.05$) was used to indicate the significance.

3. Results and discussion

The two *L. plantarum* strains were found to remove microcystin-LR from water under all experimental conditions, but there were significant differences in elimination efficacy. At pH 5.0 the removal percentages of viable *L. plantarum* IS-10506 and IS-20506 after 30 h incubation at 22 °C (49% and 52%, respectively) were lower than those at pH 7.0 (75% and 81%, respectively). Thus, further experiments were carried out at pH 7.0.

L. plantarum is a mesophilic microorganism with an optimum growth temperature ranging between 20 and 35 °C (De Angelis et al., 2004; Patrignani et al., 2006), a temperature range within which most food fermentations with dadih are carried out.

The viable cells of *L. plantarum* IS-10506 (8.6×10^{10}–1.2×10^{11} cfu per assay) after 30 h incubation at 22 °C removed 75 % of 100 μg/L MC-LR. Inactivation by heat treatment (100 °C, 30 min) as well as by exposure to HCl 1 N for 20 min resulted in a significantly decreased removal ability towards MC-LR, being as low as 11% and 5%, respectively, after 30 h incubation (Fig. 1A). The viable cells of *L. plantarum* IS-20506 (7.6×10^{10}–1.6×10^{11} cfu per assay) after 30 h incubation at 22 °C in PBS (pH 7) removed 81% of 100 μg/L MC-LR. The non-viable cells inactivated by heat treatment or HCl also showed significant reduction of removal ability towards MC-LR, 23% and 10%, respectively, after 30 h incubation (Fig. 1B).

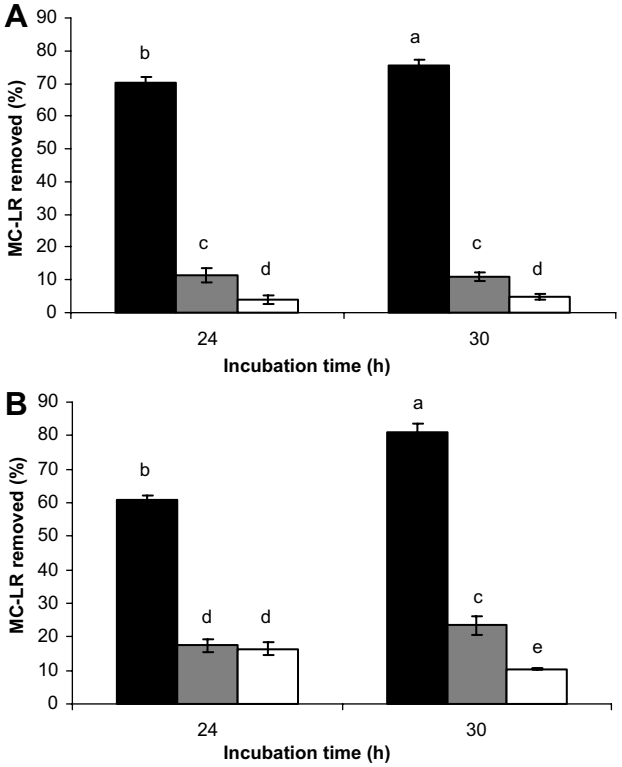

Fig. 1. (A) Microcystin-LR removal percentage of viable (black), heat-treated (grey), and acid treated (white) cells of *Lactobacillus plantarum* IS-10506, 8.6×10^{10}–1.2×10^{11} cfu per assay at pH 7, 22 °C. Error bars show the SD of the mean of three experiments. Bars with different letter are significantly different at 95% confidence level. (B) Microcystin-LR removal percentage of viable (black), heat-treated (grey), and acid treated (white) cells of *Lactobacillus plantarum* IS-20506, 7.6×10^{10}–1.6×10^{11} cfu per assay at pH 7, 22 °C. Error bars show the SD of the mean of three experiments. Bars with different letter are significantly different at 95% confidence level.

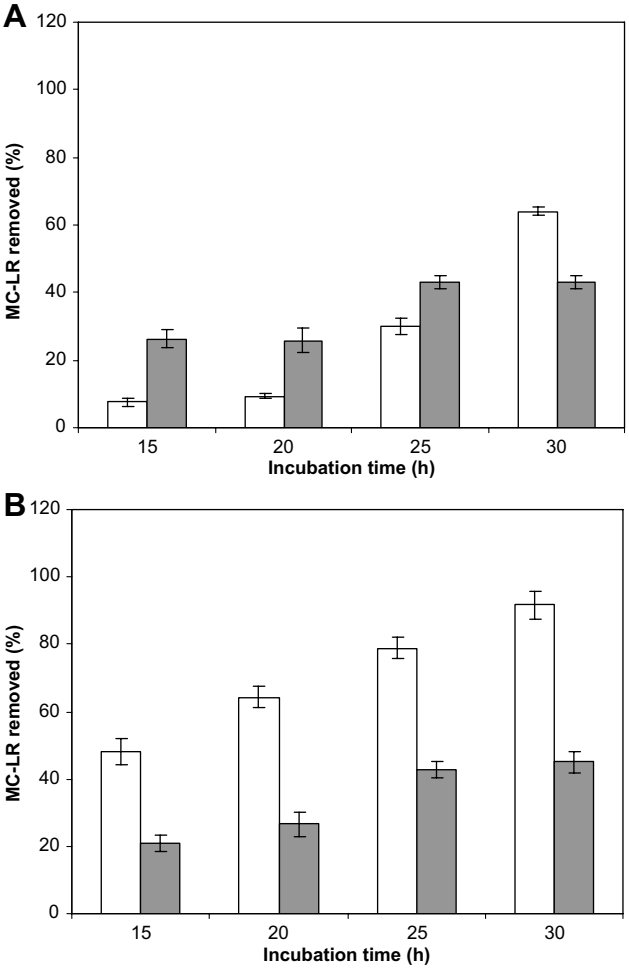

Fig. 2. (A) Microcystin-LR removal percentages by viable cells of *Lactobacillus plantarum* IS-10506 at pH 7, white bars indicate 22 °C and grey bars 37 °C. Error bars indicate confidence intervals at 95% level. (B) Microcystin-LR removal percentages of viable cells of *Lactobacillus plantarum* IS-20506 at pH 7,white bars indicate 22 °C and grey bars 37 °C. Error bars indicate confidence intervals at 95% level.

Viable cells of both *L. plantarum* strains showed significantly higher removal abilities as compared to inactivated cells, and the longer incubation time, 30 h, demonstrated higher removal percentage of MC-LR as compared to 24 h.

L. plantarum strains IS-10506 and IS-20506 showed MC-LR removal performances at both 22 and 37 °C (Figs. 2A and B), but MC-LR removal at 22 °C was higher after 30 h incubation. The viability of *L. plantarum* decreased at 37 °C in the presence of glucose, but faster when the cells were incubated in an increasing concentration of glucose (0%, 1%, 2% and 3%, w/v). Moreover, the presence of MC-LR resulted in a more pronounced decrease of viability at a higher glucose concentration. At 37 °C, the metabolic rate is high and the viable cells will become exhausted as shown by the rapid decrease of viability at 30 h incubation.

Glucose supplies energy to microbial cells at stationary phase. Preference of *L. plantarum* towards glucose has been suggested by Samuel et al. (1980) and Gobbetti et al. (1994). During the stationary phase, *L. plantarum* continued to consume glucose and produce additional ATP for cell maintenance and survival. As a consequence *L. plantarum* produces lactic acid which in turn causes cell stress as *L. plantarum* strains are unable to withstand the low pH for a long time (Venkatesh et al., 1993; Passos et al., 1994; Charalampopoulos et al., 2002).

This phenomenon was observed in our study, in the presence of 2 and 3% glucose, cells took up toxin faster and died faster, shown by a 2–3 log cycles decrease of viability over 30 h incubation. When no glucose or 1% glucose was used in the test solution the decrease in viability was less than 1 log cycle. Thus, we suggest that microcystin could be either directly toxic to *L. plantarum* or its elimination could exhaust the cells. No eliminated toxin was released by non-viable cells.

In a pilot study, the viable count of bacterial cells was conducted at 37 °C by plate counting (yielding cfu/mL) as well as by LIVE/DEAD BacLight Bacterial Viability Stain Kit (Molecular Probes), using a fluorescence activated cell sorter flow cytometer (FACS) (yielding viable cells/mL). It was observed that both strains of *L. plantarum*

maintained stable viability in PBS (pH 7.0) for the first 9 h at 37 °C , while in the presence of 1% glucose the amount of viable cells in both *L. plantarum* strains decreased by 5% and 9%, respectively. At 22 °C, the viability remained stable with or without glucose. Extended incubation times of viable bacteria with MC-LR at 37 °C in the presence of glucose led to a further decrease in viability verified by both plate counting and flow cytometer.

Both strains of *L. plantarum* from dadih behaved in a similar fashion in response to glucose supplementation, with faster and higher removal ability of MC-LR at 37 °C (Figs. 3A and B). The two strains removed MC-LR at comparable rates expressed as ng by viable cells per hour, but IS-20506 somewhat faster than IS-10506. The most efficient removal was always observed with 1% glucose supplementation, and high toxin removal was achieved, i.e. 52 ng/h for 10^{12} viable cells (IS-10506, at 25 h) and i.e. 56 ng/h for 10^{12} viable cells (IS-20506, at 20 h).

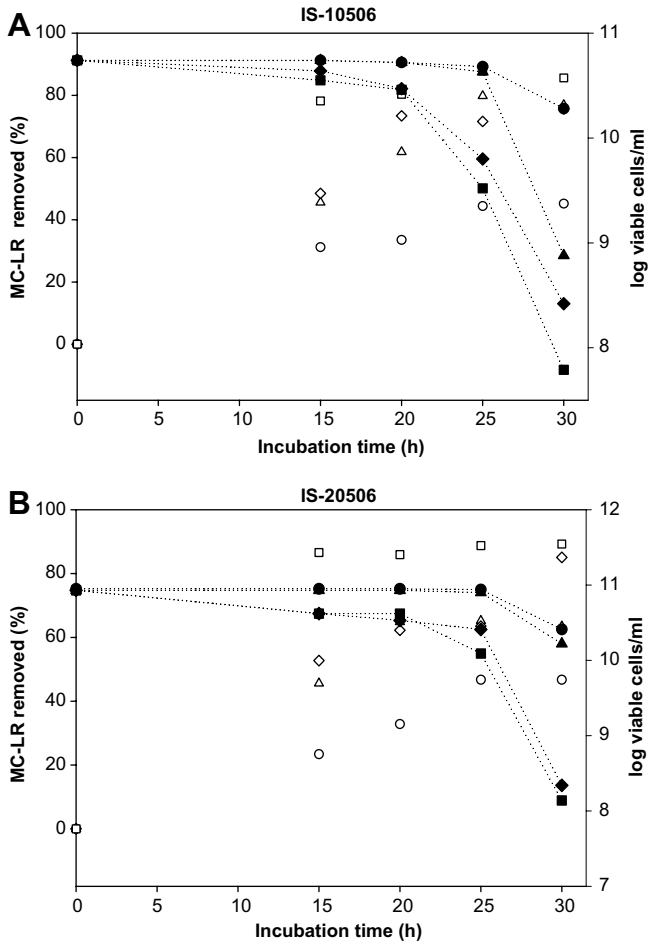

Fig. 3. (A) MC-LR removed (%) and log viable cells/mL of IS-10506 strain at different glucose concentration during incubation at 37 °C. Empty symbols stand for MC-LR removed, and filled symbols for log viable cells. Symbols (○, ●) denote 0% of glucose, (▲, Δ) 1% of glucose, (◆, ◇) 2% of glucose and (■, □) 3% of glucose. (B) MC-LR removed (%) and log viable cells mL^{-1} of IS-20506 strain at different glucose concentration during incubation at 37 °C. Empty symbols stand for MC-LR removed, and filled symbols for log viable cells. Symbols (○, ●) denote 0% of glucose, (▲, Δ) 1% of glucose, (◆, ◇) 2% of glucose and (■, □) 3% of glucose.

The results of this study demonstrate that cell viability and metabolic activity play important roles in microcystin-LR removal. Lower removal ability of *L. plantarum* IS-10506 and IS-20506 at 37 °C compared to at 22 °C is supposed to be due to lack of energy supply in PBS only. High removal percentage of both strains *L. plantarum* IS-10506 and IS-20506 at 22 °C might be also due to the optimal temperature of the enzyme activity involved.

A decrease in bacterial culturability (log cfu/mL) of *L. plantarum* IS-10506 was observed during the extended incubation (15 h) in the presence of 3% glucose. The obtained results (Figs. 3A,B) demonstrated that the longer the incubation time with MC-LR, the less viable *L. plantarum* IS-10506 and IS-20506 become in the presence of glucose. A higher glucose percentage led to a faster reduction in viability and culturability (data not shown). The striking difference between plate count results and flow cytometry results indicates that a sub population of *L. plantarum* strains is possibly in an active but non-culturable stage. Interestingly, the removal capability remains high. Thus, the viable but non-culturable cells were still actively involved in removing microcystin-LR. In the presence of microcystin-LR, supplementation with glucose resulted in more decreased viability besides high removal ability. This finding also confirms that the cells may become exhausted or suffer from the toxin exposure. In response to energy starvation or stress conditions, many bacteria convert to a viable but non-culturable condition (VBNC) rather than die and lyse. Bacteria that have reached the VBNC state are unable to grow in conventional media, but still maintain their membrane integrity and indicators of metabolic activity, but they lose the ability to grow in normal culture media. (Kjelleberg, 1993; Barcina et al., 1997; Kell et al., 1998; Colwell, 2000; Lowder et al., 2000; Pruzzo et al., 2003). Our results confirm the assumption that metabolic activity is maintained functional in *L. plantarum* strains entering the VBNC state at stationary phase, as shown by higher and faster removal of MC-LR in the presence of glucose.

Degradation of MC-LR by gram-negative bacteria, *Sphingomonas* sp., *Paucibacter toxinivorans*, *Sphingosinicella microcystinivorans*, and *Pseudomonas aeruginosa* has been reported (Tsuji et al., 2006; Park et al., 2001; Valeria et al., 2006; Bourne et al., 1996; Rapala et al., 2005; Maruyama et al., 2006; Takenaka and Watanabe, 1997). The long history of safe use of lactic acid bacteria including *L. plantarum* in foods has an advantage when selecting food grade bacteria for removing MC-LR.

The efficient toxin removal by *L. plantarum* strains IS-10506 and IS-20506, two candidate probiotics, may offer new facile tools for microcystin decontamination. In developing countries, where risk management strategies for cyanobacteria and their toxins have not been applied yet, the microcystin removal ability of probiotics may contribute to reducing the health risk caused by toxic microcystins.

The high toxin removal ability of *L. plantarum* IS-10506 and IS-20506 at 37 °C in the presence of glucose makes dadih made with these strains a promising functional food with potential probiotic properties.

Further studies are necessary to characterize the active microcystin removal properties of viable *L. plantarum* IS-10506 and IS-20506 in detail. A key question is the nature and toxicity of intermediate and final metabolites. All enzymatic and chemical modifications of microcystins reported thus far have had lower toxicity than the intact microcycstins. We anticipate that the microcystin elimination by lactobacilli will conform to this pattern.

4. Conclusions

High microcystin-LR removal by viable *L. plantarum* from dadih was observed under most studied condition.

The most efficient toxin removal of the applied toxin dose of 100 μg/L, 56 ng/h by 10^{12} viable cells was achieved with 1% (w/v) glucose supplementation at 37 °C, after 20 h incubation, providing evidence for active metabolic elimination of microcystin-LR by viable *L. plantarum* IS-20506 in the presence of 1% glucose.

Conflict of interest statement

All authors state that there are none.

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