

# REMOVAL OF CYANOBACTERIAL TOXINS BY STRAINS OF PROBIOTIC BACTERIA

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The thesis is based on the following original publications, which are referred to in the text by Roman numerals (I–IV).

- I. Nybom S.M.K., Salminen S.J., Meriluoto J.A.O. 2007. Removal of microcystin-LR by strains of metabolically active probiotic bacteria. *FEMS Microbiology Letters* 270: 27–33.
- II. Nybom S.M.K., Salminen S.J., Meriluoto J.A.O. 2008. Specific strains of probiotic bacteria are efficient in removal of several different cyanobacterial toxins from solution. *Toxicon* 52: 214–220.
- III. Nybom S.M.K., Collado M.C., Surono I.S., Salminen S.J., Meriluoto J.A.O. 2008. Effect of glucose on the removal of microcystin-LR by viable commercial probiotic strains and strains isolated from dadih fermented milk. *Journal of Agricultural and Food Chemistry* 56: 3714–3720.
- IV. Nybom S.M.K., Dziga D., Heikkilä J.E., Kull T.P.J., Salminen S.J., Meriluoto J.A.O. 2012. Characterization of microcystin-LR removal process in the presence of probiotic bacteria. *Toxicon* 59: 171–181.

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## CONTRIBUTION OF THE AUTHOR

The experimental work was planned and the manuscripts prepared by the author under the supervision of docent Jussi Meriluoto and Professor Seppo Salminen. All laboratory work was carried out by the author with the following exceptions:

In publication III, the flow cytometric analyses and the microscopic studies were performed together with Dr Maria Carmen Collado. The toxin-removal studies for the Indonesian strains were performed together with Dr Ingrid Surono.

In publication IV, the enzyme assay and proteinase experiments were carried out by Dr Dariusz Dziga. The radiolabelled experiments and toxin-removal studies for *Sphingomonas* strains were performed together with Dr Jari Heikkilä.

Purified toxins were provided by Dr Lisa Spoof to all experiments.

The author of this thesis was the principal author of all four publications.

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## ABBREVIATIONS

ACN	Acetonitrile
Adda	3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4( <i>E</i> ),6( <i>E</i> )-dienoic acid
AFB <sub>1</sub>	Aflatoxin B <sub>1</sub>
Arg	Arginine
Asp	Aspartic acid
<i>B.</i>	<i>Bifidobacterium</i>
bw	Body weight
CFU	Colony forming unit
CYN	Cylindrospermopsin
Cys	Cysteine
DAD	Diode-array detector
dm	Demethyl
dw	Dry weight
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
Glu	Glutamic acid
GRAS	Generally recognised as safe
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IDF	International Dairy Federation
i.p.	Intra-peritoneal
IT	Ion trap
i.v.	Intra-venous
<i>L.</i>	<i>Lactobacillus</i>
LC-MS	Liquid-chromatography-mass spectrometry
LD <sub>50</sub>	Dose that is lethal to 50% of test organisms
Leu	Leucine
LPS	Lipopolysaccharide
Mdha	N-methyldehydroalanine
Mdhb	2-(methylamino)-2-dehydrobutyric acid
MeOH	Methanol
MC	Microcystin
MRS	deMan-Rogosa-Sharpe broth
MW	Molecular weight
<i>m/z</i>	Mass to charge ratio
NOD	Nodularin
PBS	Phosphate buffered saline
PMSF	Phenylmethanesulfonyl fluoride
PPIA	Protein phosphatase inhibition assay

QPS	Qualified Presumption of Safety
RP	Reversed phase
RT	Retention time
SIR	Single ion recording
TFA	Trifluoroacetic acid
UV	ultra violet
VBNC	Viable but non-culturable
WHO	World Health Organization

## 1. REVIEW OF THE LITERATURE

### 1.1 Introduction

Water is essential for life, and providing the human population with safe drinking water is one of the most important issues in public health. Cyanobacteria (blue-green algae) produce toxins that may present a hazard for drinking water safety. These toxins (microcystins, nodularins, saxitoxins, anatoxin-a, anatoxin-a(S), cylindrospermopsin) are structurally diverse and their effects range from liver damage, including liver cancer, to neurotoxicity. The occurrence of cyanobacteria and their toxins in water bodies used for the production of drinking water causes a technical challenge for water treatment and cleaning. Toxic cyanobacteria in water used for recreational purposes pose a hazard to humans. Blooms of cyanobacteria accumulating along shores also present a hazard to wild and domestic animals.

The presence of toxins in drinking water creates a potential risk of toxin exposure for water consumers. Conventional water treatment procedures (such as coagulation, flocculation, clarification and sand filtration) are normally efficient methods of removing cyanobacterial cells, but are not effective in removing or destroying dissolved cyanobacterial toxins. Besides the chemical and physical methods used, biological degradation could be an efficient method of water detoxification.

Specific strains of *Lactobacillus* and *Bifidobacteria* have been empirically used in the production of fermented dairy foods, in the preservation of food and drinks, and as probiotics. Lactic acid bacteria have a long history of safe use in food and food fermentations, and normal healthy intestinal microbiota also contains many strains of lactic acid bacteria. The previously-reported capacity of lactic acid bacteria and bifidobacteria in removal of different complex contaminants led to the hypothesis of using such food grade bacteria as a possible new and safe method of water decontamination of cyanobacterial toxins.

### 1.2 Cyanobacteria

Cyanobacteria, also known as blue-green algae, have a long evolutionary history and are among the oldest organisms in the world. There is evidence of the organisms even from around 3500 million years ago (Schopf, 1993). Cyanobacteria carry out oxygen-evolving photosynthesis. The earth's oxygen-rich atmosphere and the cyanobacterial origin of plastids in plants are the two major evolutionary contributions made by cyanobacteria. Certain cyanobacteria are able to carry out nitrogen fixation.

Cyanobacteria occur in various environments including water, such as fresh and brackish water, oceans, hot springs, moist terrestrial environments such as soil, glaciers and in symbioses with plants, lichens and primitive animals. These organisms are often the first to colonise bare areas. They can also tolerate extremely high and low temperatures (Castenholz, 1973). In aquatic

environments, cyanobacteria are important primary producers and form a part of the phytoplankton. They may also form biofilms and mats (benthic cyanobacteria). In eutrophic water, cyanobacteria frequently form mass occurrences, so-called water blooms. Mass occurrences of cyanobacteria can be toxic. They have caused a number of animal poisonings and may also pose a threat to human health.

Cyanobacteria are small unicellular or filamentous bacteria with some characteristics of algae, such as the cell wall structure, pigments like chlorophyll-a and phycobiliproteins, 70S ribosomes, and the ability to perform oxygenic photosynthesis (Chorus, 2001), although they have no membrane bound nucleus or sub-cellular organelles. They can multiply only asexually by binary fission, forming endospores. The photosynthetic pigments are located in thylakoids and vary from blue-green to violet-red. Cyanobacteria have a well-developed ability to store essential nutrients and metabolites, such as glycogen granules, lipid globules, cyanophycin granules and carboxysomes, under favourable conditions.

Cyanobacteria produce many different classes of biologically active compounds, including hepatotoxic cyclic peptides, microcystins and nodularins, cytotoxic cylindrospermopsins, neurotoxic anatoxin-a and -a(S), saxitoxins, neurotoxic amino acid  $\beta$ -N-methylamino-L-alanine (BMAA) and non-toxic irritating lipopolysaccharides (reviewed in Sivonen and Jones, 1999; Meriluoto and Codd, 2005; Hudnell, 2008), see sections 1.4.1–1.4.6. Although both neurotoxins and hepatotoxins are distributed worldwide (Carmichael, 1992 and 1994), it appears that hepatotoxic blooms of cyanobacteria are more commonly found than neurotoxic blooms, and neurotoxins are considered to be of lower risk as they are less stable (Fawell *et al.*, 1994). In contrast, hepatotoxins are highly stable and exposure to these toxins has resulted in significant toxicity to both animals and humans.

Cyanobacteria are ubiquitous in their distribution in both fresh and marine waters. Toxic cyanobacterial blooms have been reported in most parts of the world (reviewed in Chorus and Bartram, 1999) (Table 1.1). Cyanobacterial blooms are a result of the increasing eutrophication in waterbodies (Chorus and Bartram, 1999). Most of these cyanobacteria are harmful to animals and humans because of their production of toxins.

### **1.3 Conditions of cyanobacterial bloom formation**

Over the past several centuries, human nutrient over enrichment in water, particularly nitrogen and phosphorus, associated with urban, agricultural and industrial development, has promoted eutrophication, which favours algal and cyanobacterial bloom formation. Decay of these excessive blooms results in decreased dissolved oxygen and the release of cyanotoxins in the water, which can result in mortality of animals and even humans (Chorus and Bartram, 1999). Prevention of bloom formation is therefore the most efficient method for avoiding cyanobacterial toxin contamination of drinking water.

Most cyanobacteria are planktonic, while some attach to sediments or surfaces and are benthic, others occur as single cells or filaments and colonies. Some cyanobacteria accumulate near the surface as blooms or scums and their growth is favoured by optimal pH, temperature, humidity, nitrogen, phosphorous, salinity, micronutrients, turbidity and light (Utkilen and Gjølme, 1992). Rain, heavy winds or cooler temperatures often inhibit growth or break up the bloom, mixing it into the water body. However, under continuing favourable conditions blooms may last for several weeks.

Cyanobacteria require low energy to maintain cell function and structure. While high turbulence and high water flows are unfavourable, macrophytes compete with cyanobacteria for nutrients and light, suppressing their growth. High temperature offers competitive advantage to cyanobacteria over phytoplankton. The pigments in cyanobacteria use the green, yellow and orange wavelengths of the spectrum (500–650 nm), which are hardly used by phytoplankton. Most cyanobacteria are sensitive to high light intensities, but those that form blooms are more resistant (Paerl and Paul, 2011).

Cyanobacterial water blooms can be observed as greenish scum (or red-brown in the case of red-pigmented strains) floating in surface water. However, some cyanobacteria, such as *Cylindrospermopsis* and *Planktothrix*, bloom in deeper water layers and may not produce obvious blooms. Masses of floating scum prevent the use of water for recreation and may spoil the taste of water because of strongly odorous metabolites. Poisonings caused by the cyanotoxins produced during heavy blooms have affected both humans and wild and domestic animals. Both hepatotoxic and neurotoxic poisonings have been associated with mass occurrences of cyanobacteria (Carmichael, 1997; Chorus and Bartram, 1999).

### **1.3.1 Toxin release**

Most of the cyanotoxins remain in the cell until the cell is lysed. If high extracellular toxin concentrations are present in the raw water, problems will occur for drinking water treatment plants. Under natural circumstances high toxin concentrations appear during the breakdown of a cyanobacterial bloom. Cyanobacterial cells are also lysed in the presence of chemicals, such as potassium permanganate or chlorine (Kenefick *et al.*, 1993; Romanowska-Duda *et al.*, 2001), which can inhibit bacterial cell wall synthesis and enzymatic reactions. Copper sulphate also leads to a lysis of cyanobacterial cells, which is followed by the release of the intracellular toxins into the water (Kenefick *et al.*, 1993; Jones and Orr, 1994; Hawkins *et al.*, 2001). The use of these kinds of chemicals to inhibit cyanobacterial growth and clean the water has led to several intoxications of livestock. A case of severe intoxication of humans was reported in Australia after treatment of the drinking water dam with copper sulphate (Bourke *et al.*, 1983).

**Table 1.1.** Examples of distribution of cyanobacterial toxins in different parts of the world

Country	Year(s) of research	Toxins	Reference
Algeria	2001	MCs	Bouaïcha and Nasri, 2004; Nasri <i>et al.</i> , 2004
Argentina	1998–2000	MCs	Conti <i>et al.</i> , 2005
Australia	1991, 1995–2001	MCs, NOD, CYN, saxitoxins	Jones <i>et al.</i> , 1994 and 1995; Negri <i>et al.</i> , 1995; Hawkins <i>et al.</i> , 1997; Kankaanpää <i>et al.</i> , 2005
Brazil	1995–97	MCs, saxitoxins	Lagos <i>et al.</i> , 1997; Hirooka <i>et al.</i> , 1999
Canada	1990	MCs	Harada <i>et al.</i> , 1991; Kotak <i>et al.</i> , 1993
Chile	1998	MCs	Neumann <i>et al.</i> , 2000
China	1988, 2003	MCs	Zhang <i>et al.</i> , 1991; Chen and Xie, 2005
Denmark	1992–95	MCs, anatoxin-a(S)	Christoffersen, 1996; Henriksen and Moestrup, 1997; Onodera <i>et al.</i> , 1997a
Finland and Baltic sea	1989, 1994–95, 1985–87	MCs, anatoxin-a, NOD	Sivonen <i>et al.</i> , 1990, Lahti <i>et al.</i> , 1997; Sivonen <i>et al.</i> , 1989
France	1994	MCs	Vezie <i>et al.</i> , 1997
Germany	1992, 1995–96, 1999, 2000	MCs, anatoxin-a	Fastner <i>et al.</i> , 1995 and 1999; Ueno <i>et al.</i> , 1996; Bumke-Vogt <i>et al.</i> , 1999; Chorus, 2001
Greece	1987	MCs	Lanaras <i>et al.</i> , 1989
Japan	1988–92, 1992–95, 1998–2000	MCs, anatoxin-a, CYN	Watanabe <i>et al.</i> , 1992; Park <i>et al.</i> , 1993a and b; Tsuji <i>et al.</i> , 1996; Ueno <i>et al.</i> , 1996; Harada <i>et al.</i> , 2004; Ozawa <i>et al.</i> , 2005
Kenya	1998–2000	MCs	Mwaura <i>et al.</i> , 2004
Netherlands	2000–02	MCs	Janse <i>et al.</i> , 2005
Poland	2002	MCs	Mankiewicz <i>et al.</i> , 2005
Portugal	1989–92	MCs	Vasconcelos <i>et al.</i> , 1996
South Africa	1985–86	MCs	Wicks and Thiel, 1990
Spain	2000	MCs	Moreno <i>et al.</i> , 2004; Aboal and Puig, 2005
Turkey	2000–03	MCs	Albay <i>et al.</i> , 2005
UK	1990–92	MCs, anatoxin-a	Edwards <i>et al.</i> , 1992; Codd <i>et al.</i> , 1995
USA	1993, 2000–01	MCs, saxitoxins	McDermott <i>et al.</i> , 1995; Carmichael <i>et al.</i> , 1997; Onodera <i>et al.</i> , 1997b; Graham <i>et al.</i> , 2004

#### 1.4 Cyanobacterial toxins

The conditions leading to cyanobacterial proliferation are well established, but the physiological or biochemical functions of the cyanobacterial toxins are largely unknown. Cyanobacteria produce a variety of toxins that are classified functionally into hepato-, neuro-, and cytotoxins. Additionally, cyanobacteria produce lipopolysaccharides as well as secondary metabolites that might be useful pharmacologically. Most of the cyanotoxins originate from planktonic cyanobacteria, which occur in fresh and brackish water. Toxic strains of cyanobacteria have also been isolated from benthic environments. The following sections describe toxins produced by cyanobacteria more in detail.

Detection methods for cyanobacterial toxins include biochemical methods, such as nucleic acid based methods, enzyme-linked immunosorbent assay (ELISA) and protein phosphatase inhibition

assay (PPIA), and physico-chemical methods, such as high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) (reviewed e.g. in van Apeldoorn *et al.*, 2007). The development of methods for cyanotoxin analysis has not been the aim of this thesis and therefore the methods are not described more in detail in the thesis.

#### 1.4.1 Microcystins

Globally, the most frequently reported cyanobacterial toxins are cyclic heptapeptide hepatotoxins, microcystins. These can be found primarily in some species of the freshwater genera *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, and *Anabaenopsis*. Microcystins are named after *Microcystis aeruginosa*, the cyanobacterium in which the toxin was first isolated and described (Carmichael *et al.*, 1988).

Microcystins are cyclic heptapeptides with variable amino acids and a general structure of cyclo(-D-Ala<sup>(1)</sup>-L-X<sup>(2)</sup>-D-MeAsp(iso-linkage)<sup>(3)</sup>-L-Z<sup>(4)</sup>-Adda<sup>(5)</sup>-D-Glu(iso-linkage)<sup>(6)</sup>-Mdha<sup>(7)</sup>), in which amino acid residues at 2 and 4 are variable L-amino acids, D-MeAsp is D-erythro- $\beta$ -methylaspartic acid, and Mdha is N-methyldehydroalanine, while the amino acid Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (Fig. 1.1). The Adda component of microcystins is contributing to their toxicity (Carmichael, 1992; Ressom *et al.*, 1994). There are around 100 structural variants of microcystins described in the literature (listed in Sivonen and Jones, 1999; Spoof, 2004; Neffling, 2010), formed by variation in all 7 amino acids, primarily the L-amino acids at position 2, 3, 4, or 7. The main structural differences between microcystin variants such as -LR, -LA, -RR, and -YR are the substitution of single amino acids.

The most widely-distributed (Carmichael, 1992) and studied microcystin variant is microcystin-LR (MC-LR), with the amino acid residues leucine and arginine in positions 2 and 4, respectively. MC-LR is produced as a secondary metabolite mostly by *Microcystis* species as well as other cyanobacteria (Bishop *et al.*, 1959; Dittmann *et al.*, 1997; Nishizawa *et al.*, 1999). It is a water-soluble and a non-volatile toxin with a molecular weight of 994. Production of MC-LR is dependent on various factors like strain specificity, genetic differences, metabolic processes required for toxin production and can differ temporally and spatially (Ressom *et al.*, 1994). A single bloom can have both toxigenic and non-toxigenic strains within it, and the extent of toxicity depends on the dominance of toxic strains within the bloom (Chorus and Bartram, 1999). The toxins are generally bound to the cell membrane and are released as cells age and die, and under stress. They can also passively leak out of cells or be released by lytic bacteria (Carmichael, 1992).

MC-LR is hepatotoxic and is a potent tumour promoter. The primary target organ of MC-LR is the liver (Falconer *et al.*, 1983; Runnegar and Falconer, 1986; Brooks and Codd, 1987; Meriluoto *et al.*, 1990; Nishiwaki *et al.*, 1994) although it also affects the kidney, gastrointestinal tract and colon (Bell and Codd, 1994). Microcystins are taken up into the cells through the organic anion transporting polypeptides (Feurstein *et al.*, 2009), formerly called the bile acid transport system

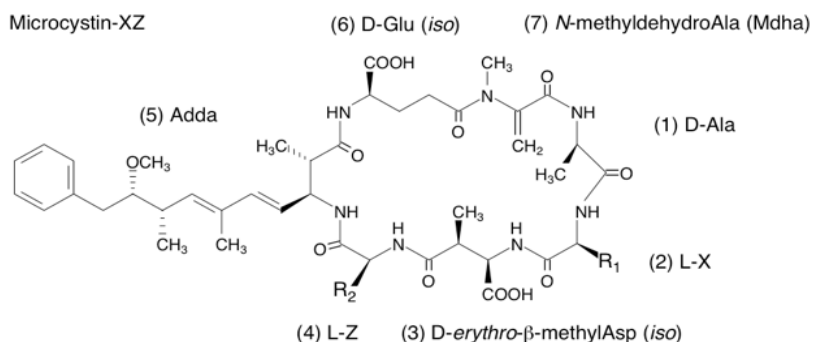


(Falconer and Yeung, 1992; Ito *et al.*, 2000), as they are unable to penetrate the cell membrane (Eriksson *et al.*, 1990). The more lipophilic microcystin variants, such as MC-LW and MC-LF, have been shown to interact more efficiently with a model membrane than MC-LR, suggesting more interactions with cell membranes (Vestervik and Meriluoto, 2003). In the liver, MC-LR induces cytoskeletal damage, disruption of the hepatic structure, necrosis and haemorrhage (Beasley *et al.*, 2000; Hooser, 2000). MC-LR also results in liver enlargement, decreased systemic arterial blood pressure and eventually death (Beasley *et al.*, 2000).

Microcystins are potent and specific inhibitors of serine/threonine-specific protein phosphatases 1 and 2A (Mackintosh *et al.*, 1990). Inhibition of phosphatases by microcystins can alter the balance between phosphorylation and dephosphorylation, which can affect diverse biological processes (Cohen, 1989). Exposure to MC-LR has also been found to result in oxidative stress, cytoskeletal damage, cytokine induction, mitochondrial alteration, and tumour promotion (Falconer and Yeung, 1992; Falconer and Humpage, 1996). The oral dose required to induce microcystin toxicity is higher than the intravenous or intraperitoneal dose. Yoshida *et al.* (1997) found that the intraperitoneal LD<sub>50</sub> of MC-LR in mice was 65.4 µg/kg body weight (bw) and the oral LD<sub>50</sub> was 10.9 mg/kg bw. Microcystin variants differ in toxicity, in the literature reported values range from the very potent toxin MC-LR, LD<sub>50</sub> of 50 µg/kg (mouse i.p.), to MC-RR, LD<sub>50</sub> of 600 µg/kg (Sivonen and Jones, 1999).

Microcystins are distributed in waterbodies worldwide, and the toxicity on exposure to microcystins has been reported worldwide in fish, animals and humans (reviewed in Chorus, 2001). The World Health Organization (WHO) has set a provisional drinking water guideline of 1 µg/L for MC-LR (WHO, 1998; new edition in WHO, 2011) (discussed in section 1.6.1).

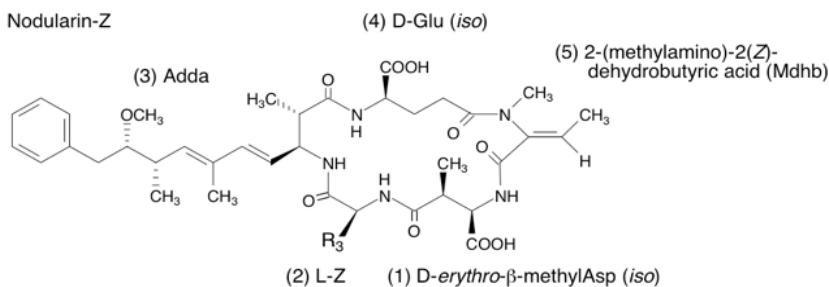
Microcystins are synthesised non-ribosomally by a multi-enzyme complex, comprised of polyketide synthases and polypeptide synthetases. These enzymes (200–2000 kDa) are composed of modules (mcyA – mcyJ), which are coded in one gene-cluster with ten open reading frames and are responsible for one synthesis step each (Neilan *et al.*, 1999; Tillet *et al.*, 2000; Rouhiainen *et al.*, 2004).



**Figure 1.1.** General structure of the hepatotoxic cyclic peptides, microcystins.

### 1.4.2 Nodularins

The cyclic pentapeptide nodularin (NOD) is common in brackish water. It occurs in the Baltic Sea as well as in saline lakes and estuaries. In the Baltic Sea, marine blooms of *Nodularia spumigena* are among some of the largest cyanobacterial mass events in the world. Nodularin has also been found in Australia. The first scientific report of the potent toxicity of cyanobacterial blooms was published in Nature by G. Francis in 1878, where the author reported that stock deaths had occurred as a result of drinking from a lake polluted by a bloom in Australia. The organism responsible for this bloom was later described as *Nodularia spumigena* and the toxin was later isolated and characterised (Rinehart *et al.*, 1988) as nodularin. The name nodularin also originates from the producer cyanobacterium, *Nodularia spumigena* (Rinehart *et al.*, 1998). Less than 10 analogues of nodularins are known (Chorus and Bartram, 1999). The chemical structure of nodularin is cyclo(D-MeAsp(iso-linkage)<sup>(1)</sup>-L-Z<sup>(2)</sup>-Adda<sup>(3)</sup>-D-Glu(iso-linkage)<sup>(4)</sup>-Mdhb<sup>(5)</sup>), in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid (Fig. 1.2). The second residue (Z) is L-Arg in the common nodularin-R, with a molecular weight of 824, and L-Val in nodularin-V isolated from the marine sponge *Theonella* (de Silva *et al.*, 1992). A few naturally occurring variants of nodularins have been found (Namikoshi *et al.*, 1994).



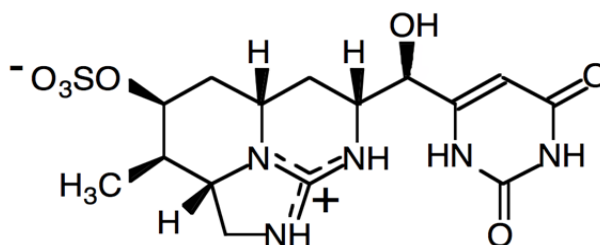
**Figure 1.2.** General structure of nodularins.

### 1.4.3 Cylindrospermopsin

Cylindrospermopsin (CYN), originally isolated from the cyanobacterium *Cylindrospermopsis raciborskii*, is an alkaloid cytotoxin with the structure of a tricyclic guanidine moiety attached to a hydroxymethyluracil (Ohtani *et al.*, 1992) and a molecular weight of 415 (Fig. 1.3). It is also known to be produced by other cyanobacteria, such as *Aphanizomenon ovalisporum* and *Anabaena bergii*. Cylindrospermopsin inhibits protein synthesis and mainly affects the liver (Terao *et al.*, 1994), but can also affect the kidney, spleen, thymus, and heart. It is a cyanotoxin occurring in tropical or subtropical regions that has recently been detected also in temperate regions. Cylindrospermopsin-producing cyanobacteria have been reported e.g. in Australia (Ohtani *et al.*, 1992; Shaw *et al.*, 1999), Japan (Harada *et al.*, 1994), Israel (Banker *et al.*, 1997), New Zealand (Stirling and Quilliam, 2001) and Finland (Spoof *et al.*, 2006). A similar guideline value as for MC-

LR, a 1 µg/L guideline value for cylindrospermopsin in drinking water has been proposed by Humpage and Falconer (2003).

New structural variants of cylindrospermopsin have been isolated (Li *et al.*, 2001; Mazmouz *et al.*, 2010). One variant was identified as deoxycylindrospermopsin by Norris *et al.* (1999), and was reported to be nearly non-toxic. Banker *et al.* (2000) elucidated the chemical structure of 7-epicylindrospermopsin and it was shown to be toxic. The uracil moiety is important for the toxicity of cylindrospermopsin.



**Figure 1.3.** Structure of cylindrospermopsin.

#### 1.4.4 Neurotoxins

Cyanobacterial neurotoxins belong to a diverse group of heterocyclic compounds called alkaloids. Three types of cyanobacterial neurotoxins, anatoxin-a, anatoxin-a(S), and saxitoxins, are known. A mild neurotoxin, BMAA, has been found in a variety of cyanobacteria (Cox *et al.*, 2005; Metcalf *et al.*, 2008).

Anatoxin-a is a small alkaloid with a molecular weight of 165, a secondary amine with the structure 2-acetyl-9-azabicyclo(4-2-1)non-2-ene (Devlin *et al.*, 1977). It mimics the effect of acetylcholine and causes rapid death by respiratory arrest. Homoanatoxin-a (MW 179) is an anatoxin-a homologue. It has a propionyl group at C-2 instead of the acetyl group found in anatoxin-a and has been found in *Oscillatoria formosa* (Skulberg *et al.*, 1992).

Anatoxin-a has been shown to be produced by several *Anabaena*, *Aphanizomenon*, *Oscillatoria* and *Planktothrix* species (reviewed in Sivonen and Jones, 1999). Anatoxin-a is perhaps the most common cyanobacterial neurotoxin, especially in North America and Europe, and has caused numerous animal poisonings.

Anatoxin-a(S) is a phosphate ester of a cyclic *N*-hydroxyguanine, originally identified from *Anabaena flos-aquae* strain NRC 525-17 (Matsunaga *et al.*, 1989). The molecular weight is 252. Anatoxin-a(S) is an irreversible acetylcholine esterase inhibitor and its characteristic signs of poisonings in mice include salivation. Anatoxin-a(S) was first reported in North America where it has caused animal poisonings and later also in Denmark (Onodera *et al.*, 1997a).

Saxitoxins, also known as paralytic shellfish poisons (PSP toxins) were originally isolated and characterised from marine dinoflagellates (Anderson, 1994). Saxitoxins are sodium channel blocking agents causing paralysis and have caused human poisonings due to their ability to concentrate in shellfish (Anderson, 1994). In cyanobacteria, saxitoxins have been found in *Anabaena circinalis*, *Anabaena lemmermannii*, *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii* and *Lyngbya wollei* (Humpage *et al.*, 1994; Onodera *et al.*, 1997b; Pereira *et al.*, 2000).

#### **1.4.5 Lipopolysaccharides**

Lipopolysaccharide (LPS) endotoxins are generally found in the outer membrane of the cell wall of Gram-negative bacteria, also in cyanobacteria. Bacterial LPS are pyrogenic and toxic (Weckesser and Drews, 1979). It is often the fatty acid component of LPS that elicits an irritant, pyrogenic or allergenic response in humans and mammals. Cyanobacterial LPS may contribute to human health problems via exposure to mass occurrences of cyanobacteria.

#### **1.4.6 Other bioactive compounds from cyanobacteria**

Cyanobacteria have been found to be a rich source of new bioactive compounds for the biotechnological and biomedical industries (Burja *et al.*, 2001; Singh *et al.*, 2005). Cyanobacteria produce bioactive compounds that are effective both against eukaryotic cells and also against viruses, bacteria and other cyanobacteria. Many of the bioactive cyanobacterial compounds are peptides (Fujii *et al.*, 2002). Bioactive compounds may have various biological activities in test animals, eukaryotic or prokaryotic cells or viruses, or they may target certain enzymes. Marine environments and especially benthic cyanobacteria seem to be among the best sources of new bioactive compounds. Much interest has been focused on screening anticancer compounds.

### **1.5 Occurrence and levels of cyanobacteria and hepatotoxins**

Toxic cyanobacteria are found worldwide both in inland and coastal water environments. They are widespread and found in a diverse range of environments, including soils, seawater and freshwater environments. Some environmental conditions, including sunlight, warm weather, low turbulence and high nutrient levels, can promote growth. Depending on the species, this may result in green discolouration of water due to a high density of suspended cells and the formation of surface scums. Such cell accumulations may lead to high toxin concentrations.

The toxins are not actively secreted to the surrounding water; most of the toxin is intracellular in growing cells. The release of toxin occurs during senescence of the cultures and when cultures shift from growth phase to stationary and death phases. Studies with laboratory cultures have shown that more than 80% of the microcystin or nodularin is intracellular in growing cells (Codd *et al.*, 1989; Sivonen, 1990; Rapala *et al.*, 1997). Also under field conditions, the majority of microcystin is intracellular during active growth of the cells (Jones and Orr, 1994; Lahti *et al.*, 1997). Cylindrospermopsin on the other hand is also produced extracellularly and high

concentrations of dissolved toxin can be found in cyanobacterial cultures producing cylindrospermopsin (Metcalf *et al.*, 2002).

Globally it appears that hepatotoxic freshwater blooms of cyanobacteria are more commonly found than neurotoxic blooms. There are reports of hepatotoxic blooms from all continents around the world (examples found in Table 1.1). Mass occurrences of neurotoxic cyanobacteria are common in some countries and have been reported in North America, Australia and Europe, where they have caused several animal poisonings (e.g. Repavich *et al.*, 1990; Baker and Humpage, 1994; Bumke-Vogt *et al.*, 1999).

Some of the highest reported cyanotoxin concentrations in bloom samples, measured by HPLC, have been 7300 µg/g dry weight (dw) microcystin in a *Microcystis* bloom from China (Zhang *et al.*, 1991), 18000 µg/g dw nodularin in a *Nodularia* bloom from the Baltic sea (Kononen *et al.*, 1993) and 5500 µg/g dw cylindrospermopsin from Australia (Sivonen and Jones, 1999). For water treatment and public health management purposes, a more appropriate unit is often toxin concentration per litre of water, for example in relating toxin concentrations to the WHO guideline level of 1µg/L of MC-LR (described in section 1.6.1). High concentrations of microcystins per litre of water have been reported among others in Germany, up to 25000 µg/L microcystin (Chorus, 1998). The quantitative determination of toxins is mostly performed from lyophilised cultures, bloom samples or seston. Results are usually expressed as milligrams or micrograms of toxin per gram of dw.

Toxic and non-toxic strains from the same cyanobacterial species cannot be separated by microscopic identification. To confirm that a particular cyanobacterial strain produces toxins, it is important to isolate a culture of that strain, and to detect and quantify toxin concentrations in the pure culture. The use of molecular genetic methods has further lead to the development of more precise identification methods for toxic cyanobacteria.

### 1.6 Human health effects

Many cyanobacteria produce potent toxins. As reported in literature, problems caused by cyanobacteria are encountered around the world and problems related to safe drinking water production are common (reviewed in e.g. Codd *et al.*, 2005). The human health effects caused by cyanobacterial toxins vary in severity from mild gastroenteritis to severe and sometimes fatal diarrhoea, dysentery and hepatitis. Each toxin has specific properties, with individual concerns including liver damage, neurotoxicity and tumour promotion. Microcystins, including the most common variant MC-LR, are hepatotoxic and potent tumour promoters. Acute symptoms reported after exposure to microcystin-containing cyanobacteria include gastrointestinal disorders, nausea, vomiting, fever and irritation of the skin, ears, eyes, throat and respiratory tract, abdominal pain, kidney and liver damage. There are several reports of human health effects associated with ingestion of water containing microcystins, with effects ranging from

gastroenteritis (Annadotter *et al.*, 2001) to liver damage (Falconer *et al.*, 1983) and even death (Teixera *et al.*, 1993) (discussed in section 1.6.2).

Humans can be exposed to a range of cyanotoxins contained either in cyanobacterial cells or released into the water. The dissolved toxins are stable against low pH and enzymatic degradation and will therefore remain intact within the digestive tract. As microcystins do not readily penetrate the cell membrane (Eriksson *et al.*, 1990), they enter the body from the intestine via the organic anion transporting polypeptides (Feurstein *et al.*, 2009). As a consequence of active transport, the toxicity of microcystins is restricted to organs expressing a suitable transporter, such as the organic anion transporter in the cell membranes. From the blood microcystins are then concentrated in the liver as a result of active uptake by hepatocytes (Runnegar *et al.*, 1981). The toxins are covalently bound to protein phosphatases in the hepatocyte cytosol (Bagu *et al.*, 1997). Some more hydrophobic microcystin variants may cross cell membranes by other mechanisms, including diffusion (Vestervik and Meriluoto, 2003).

Microcystins act primarily through inhibition of protein serine/threonine phosphatases 1 and 2A (Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990; Runnegar *et al.*, 1993). These enzymes are essential in cellular regulation and inhibition results in a higher level of protein phosphorylation in hepatocytes, which will lead to cytoskeletal damage and haemorrhage in the liver. Furthermore, the excessive signalling may lead to cell proliferation, cell transformation and tumour promotion (Fujiki and Suganuma, 1993). In hepatocytes protein phosphatase inhibition also causes disruption of intermediate actin filaments and microtubules leading to cellular disruption (Falconer and Yeung, 1992; Wickstrom *et al.*, 1995; Khan *et al.*, 1996).

Human health problems are often associated with chronic exposure to low microcystin concentrations in poorly treated drinking water, contaminated food (such as fish, mussels and prawns) or with the consumption of algal supplements contaminated with cyanotoxins (Gilroy *et al.*, 2000; Lawrence *et al.*, 2001). Exposure routes include the oral route, through inhalation or through dermal exposure. Human exposure to MC-LR primarily occurs through ingestion of contaminated drinking water (Gupta, 1998). Accidental ingestion of contaminated water during recreation is also possible. Another route of uptake is the nasal mucosa (Chorus *et al.*, 2000). During recreational activities in cyanobacterial contaminated water bodies toxins might flow into the respiratory tract (Chorus *et al.*, 2000). Renal dialysis patients are at great risk when water used for dialysis contains contaminants such as cyanotoxins. For these patients large volumes of water are used and the route of exposure is similar to the i.v. route, which leads to a greater uptake of toxin than through oral ingestion (see section 1.6.1).

The use of water containing cyanobacterial toxins for irrigation of crops presents potential health hazards through several exposure routes, including uptake into the food chain. There are several reports on plants taking up microcystins from the irrigation water (Abe *et al.*, 1996; Kos *et al.*, 1995; Järvenpää *et al.*, 2007).

In some countries cyanobacteria are sold as dietary supplements, containing the cyanobacteria *Arthrospira (Spirulina)* and *Aphanizomenon flos-aquae*. Therefore a possible route for toxin exposure is due to consumption of cyanobacterial health food tablets (Gilroy *et al.*, 2000; Lawrence *et al.*, 2001). The dermal route may play a role during recreational use of water (swimming, canoeing etc.) (Falconer, 1996 and 1999), but due to the hydrophilic character of most microcystins, anatoxins, cylindrospermopsins and saxitoxins, an uptake via the skin is unlikely. Dermal contacts cause skin irritations, such as allergic reactions, but have not been widely reported.

### 1.6.1 Reported cases of cyanotoxin exposure

Exposure to cyanotoxins has been reported worldwide in humans and animals for over a century (Francis, 1878). Some incidents are listed in Table 1.2. Exposure most often occurs orally, but can also occur through inhalation or through skin exposure. Human exposure to MC-LR is primarily through ingestion of contaminated drinking water (Gupta *et al.*, 1998) and by recreational contact with contaminated water, by consumption of fish or cyanobacterial products from contaminated water. Even minor contact with cyanobacteria in bathing water can lead to skin irritation and increased probability of gastrointestinal symptoms (Pilotto *et al.*, 1997).

The two most lethal poisonings by cyanobacteria in drinking water have occurred in Brazil. A severe gastro-enteritis epidemic affecting about 2000 persons, of which 88 died, followed the flooding of a newly built dam reservoir (Itaparica Dam) in Brazil in 1988 (Teixeira *et al.* 1993). Both water samples from the dam and blood and faecal samples from the patients were subjected to bacteriological, virological and toxicological tests. The results showed that toxins produced by cyanobacteria present in a bloom of *Anabaena* and *Microcystis* in the dam water were responsible for the epidemic (Teixeira *et al.* 1993). In 1996, an outbreak of severe hepatitis occurred at a Brazilian haemodialysis centre in Caruaru, Brazil. At this clinic 117 of 136 patients experienced visual disturbances, nausea, vomiting, muscle weakness and painful hepatomegaly, following routine haemodialysis treatment. Later, 100 patients developed acute liver failure and 76 of the patients died. MC-LR and other microcystin congeners were found in the water and also blood and liver tissue samples from the patients were verified to contain microcystins (Pouria *et al.*, 1998; Jochimsen *et al.*, 1998; Carmichael *et al.*, 2001; Yuan *et al.*, 2006; Hilborn *et al.*, 2007). Cylindrospermopsin was also found in the clinic's filtration system (Carmichael *et al.*, 2001).

There have been many incidences of human illness through drinking water in Australia and North America, where microcystins have been present in the water (Bourke *et al.*, 1983; Falconer, 1989 and 1996). The earliest incidence of cyanotoxin exposure in North America was reported in 1931, where 5000–8000 people who consumed water from Ohio and Potomac rivers containing a *Microcystis*-bloom showed symptoms of gastroenteritis and illness (Tisdale, 1931). In 1975, endotoxic shock of 23 dialysis patients in Washington DC was attributed to cyanobacterial contamination in a drinking water reservoir (Hindman *et al.*, 1975).

The high prevalence of primary liver cancer in regions of China, where pond and ditch water are used as drinking water supplies, has been investigated and discussed by several authors, and chronic effects of long-term exposure to microcystins in drinking water have been considered (Yu, 1989; Harada *et al.*, 1996; Ueno *et al.*, 1996). Cyanobacteria are abundant in surface waters in South East China where the incidence of hepatocellular carcinoma is highest, and it has been proposed that microcystins in the drinking water are responsible for the higher incidences of cancer in persons drinking the pond and ditch water (Yu, 1995; Yu *et al.*, 2001). In China MC-LR exposure through drinking water has also been related to colorectal cancer in the population (Zhou *et al.*, 2000 and 2002).

In 1995, adverse health effects after recreational water contact in Australia were observed in 852 persons. Results showed an elevated incidence of diarrhoea, vomiting, flu-like symptoms, skin rashes, mouth ulcers, fevers, and eye or ear irritations within a week following exposure (Pilotto *et al.*, 1997). Symptoms correlated significantly with duration of water contact and cell density of cyanobacteria.

Numerous cases of animal poisoning (often lethal) after exposure to cyanobacteria have also been reported in literature. One severe mammalian poisoning incident was the deaths of sheep drinking from a farm dam contaminated with the neurotoxin saxitoxin in Australia (Negri *et al.*, 1995). There are also several other incidents that have resulted in animal poisoning and death, such as fish and bird deaths in Finland, dog deaths in the UK and 2000 deaths of livestock in Australia (Eriksson *et al.*, 1986; Pearson *et al.*, 1990; Humpage *et al.*, 1994). Codd *et al.* (2003) summarised studies of cyanotoxin-related mortalities of flamingos. In the flamingos toxins were found in intestinal tissue, but also in the feathers of the birds (Krienitz *et al.*, 2003; Metcalf *et al.*, 2006).



**Table 1.2.** Examples of incidents with cyanotoxin contamination

Location	Year	Incident	Cyanobacteria or toxin	Reference
Lake Alexandrina, Australia	1878	Deaths of sheep, horses, dogs, pigs	NOD	Francis, 1878
Ohio River, USA	1931	Humans, gastro-enteritis	<i>Microcystis</i> sp.	Tisdale, 1931
Zimbabwe	1966	Humans, gastro-enteritis	<i>M. aeruginosa</i>	Zilberg, 1966
Palm Island, Australia	1979	Humans, hepato-enteritis	CYN	Bourke <i>et al.</i> , 1983
Armidale, Australia	1981	Humans, liver damage	<i>M. aeruginosa</i>	Falconer <i>et al.</i> , 1983
Porvoo, Finland	1984	Dog deaths	NOD	Persson <i>et al.</i> , 1984
Åland Islands, Finland	1985	Fish and bird deaths	MC	Eriksson <i>et al.</i> , 1986
Richmond Lake, USA	1988	Poisoning of livestock, dogs, fish, birds	Anatoxin-a(S)	Mahmood <i>et al.</i> , 1988
England, UK	1988	Dog deaths	MC	Pearson <i>et al.</i> , 1990
Itaparica Dam, Brazil	1988	Humans, 88 deaths, gastro-enteritis	<i>Anabaena</i> sp. <i>Microcystis</i> sp.	Teixeira <i>et al.</i> , 1993
Darling River, Australia	1990–91	Livestock, 2000 deaths	Saxitoxins	Humpage <i>et al.</i> , 1994
Loch Insh, Scotland, UK	1990–91	Dog poisonings	Anatoxin-a	Edwards <i>et al.</i> , 1992
Nandong District, Jiangsu Province, Shanghai, China	1993–95	Liver cancer in population drinking surface water	MC	Yu, 1995; Ueno <i>et al.</i> , 1996
Loch Leven, Scotland, UK	1994	Fish deaths	MC	Rodger <i>et al.</i> , 1994
Scania, Sweden	1994	Humans, gastro-enteritis	MC	Annadotter <i>et al.</i> , 2001
Australia	1994	Sheep deaths	Saxitoxins	Negri <i>et al.</i> , 1995
New South Wales, Australia	1995	Health effects in 852 humans	Toxin not identified	Pilotto <i>et al.</i> , 1997
Caruaru, Brazil	1996	Hemodialysis patients, 76 deaths	MC	Jochimsen <i>et al.</i> , 1998; Pouria <i>et al.</i> , 1998; Carmichael <i>et al.</i> , 2001
North Island, New Zealand	2005	Dog deaths	Anatoxin-a	Wood <i>et al.</i> , 2007

### 1.6.2 Risk assessment

Many reported incidents of human health effects have involved inappropriate treatment of water supplies. The health risk caused by cyanotoxin exposure is difficult to quantify, since the actual exposure and resulting effects have not been conclusively determined. The most likely route for human exposure is the oral route via drinking water (Falconer, 1996 and 1999), and from recreational use of lakes and rivers (Pilotto *et al.*, 1997). The growth of cyanobacteria in lakes and rivers used for recreational purposes is today recognised as a public health issue. Water blooms of cyanobacteria may be associated with unpleasant odours and appearance of shores. As adequate surveillance for toxin production is often difficult, the users need to be alert for safe use of recreational water.

Due to the growing concern about health effects of cyanotoxins especially via drinking water, WHO has adopted a provisional guideline value of 1.0 µg/L for MC-LR in 1998 (WHO, 1998). The newest 4<sup>th</sup> edition to the drinking water guideline was published in 2011 (WHO, 2011). This guideline value is based on a tolerable daily intake (TDI) value derived from two animal studies (Falconer *et al.*, 1994, Fawell *et al.*, 1994); a mouse study and a pig study. The first study was a 13-week mouse oral study with pure MC-LR, which determined a provisional TDI of 0.04 µg/kg body weight per day for MC-LR. It was supported by a 44-day pig oral study with exposure to an extract from *M. aeruginosa* containing MC-LR in the drinking water of the animals, which resulted in a provisional TDI of 0.067 µg/kg body weight per day. The WHO guideline value was derived based on a no-observed-adverse-effect level for mice and pigs using the lower of these two values and the TDI was calculated using an uncertainty factor of 1000. This provisional guideline value is applicable only for MC-LR, since the database for other microcystin variants is too small to derive a TDI. The directive concerning the management of bathing water quality (Directive 2006/7/EC) also acknowledges the managing, monitoring and classification of bathing water quality and the provision of information to the public on bathing water quality.

Assessment of different water treatment procedures has shown that many of the treatment methods result in a reduction of cyanotoxin concentrations to below acutely toxic levels and below the WHO guideline value of 1.0 µg/L MC-LR in drinking water. During a cyanobacterial bloom the treatment procedures may however be insufficient, and also when water treatment procedures are not used in combination (such as chlorination and activated carbon). Therefore it is important to observe the water treatment efficiency during cyanobacterial blooms. Toxin levels during different steps of water treatment also have to be monitored.

Binding to and inhibition of protein phosphatases is a key mechanism by which microcystins and nodularins exhibit their toxicity. It is therefore important to know if structural modifications of the toxin molecule change the affinity to and inhibition of the phosphatase. So far, it has been shown that neither the Adda residue alone (Namikoshi *et al.*, 1989; Choi *et al.* 1993) nor linear microcystin- or nodularin-precursor peptides bind protein phosphatases (Taylor *et al.*, 1996).

### **1.7 Drinking water and water treatment**

Water is an essential natural resource, necessary for drinking, agriculture and industrial activities. Contamination of water can therefore influence humans, agricultural livestock and irrigated field crops, as well as wildlife drinking the water or living in the aquatic environment. Drinking water should be pure enough to be consumed or used with low risk of immediate or long term harm. In most developed countries, the water supplied to households and industry is of drinking water standard, even though only a small proportion is actually used as drinking water or in preparation of food. In large parts of the world, the population has inadequate access to safe potable water and use sources contaminated with disease vectors, pathogens or unacceptable levels of toxins and other harmful substances. As the world's population grows the pressure on both water

supplies and water quality increases. In many parts of the world water is a limited resource, although the water on the planet and the annual rainfall should be enough to exceed the needs.

When evaluating methods for water treatment for the removal of cyanobacterial toxins, factors relating to intracellular and dissolved toxins should be taken into account. Cyanotoxins are produced within the cyanobacterial cells and thus toxin removal involves procedures to destroy or avoid the cells. Usually microcystin release occurs late in the exponential growth phase and increases during the stationary phase (Codd *et al.*, 1989; Sivonen, 1990; Rapala *et al.*, 1997). The cyanotoxins are also water soluble and therefore chemical or biological procedures reducing the toxicity or completely removing the toxins from the drinking water are needed.

In cyanotoxin removal from drinking water there is a need for knowledge of the physical and chemical properties of the toxin, such as the hydrophobicity, molecular size, and functional groups, the nature of the toxin, i.e., intracellular or extracellular, cyanobacterial growth and bloom patterns, and effective treatment processes (Chorus and Bartram, 1999). However, these treatments may not be sufficient during cyanobacterial blooms or when a high organic load is present, and toxin levels should therefore be monitored during all steps of water treatment processes. Some of the important methods of drinking water treatment are shortly described in the following section. A more detailed review on drinking water treatment and cyanotoxin removal was recently published by Westrick *et al.* (2010).

### **1.7.1 Water treatment processes**

Conventional surface drinking water treatment utilises coagulation, flocculation, sedimentation, filtration and disinfection as basic methods. However, conventional treatment may need to be optimised for cyanotoxin removal, relating to the form of the toxin to be removed (intra- or extracellular), the background water matrix, and possible dissolved toxin release during the treatment process (Falconer, 2005).

Coagulation or flocculation involves the aggregation of smaller particles into larger particles using chemicals, such as ferric chloride or aluminium sulphate. Coagulation can be an efficient method for eliminating cyanobacterial cells from water, but soluble cyanotoxins are not very efficiently removed by this method (Rositano and Nicholson, 1994). Coagulation may also cause additional problems such as lysis of cyanobacterial cells leading to release of toxins.

The activated carbon approach uses either powdered activated carbon, which can be added occasionally when there is a need, or granular activated carbon adsorbers, which are used continuously (Newcombe, 2002). To achieve high removal efficiencies, high doses of powdered activated carbon are required and the contact time is important. Both microcystins and cylindrospermopsin can be absorbed by activated carbon (Newcombe, 2002), but microcystin variants may have different adsorption efficiencies. The disposal of the carbon containing cyanobacterial toxins may present a challenge for this type of treatment.

Rapid filtration is a method usually used after a coagulation step in conventional water treatment. Lepistö *et al.* (1994) showed that rapid filtration does not effectively remove cyanobacterial cells from water. Conventional water treatment requires regular backwashing of the filters, but if the washing process is inadequately performed, lysis of cyanobacterial cells on the filters can lead to release of toxins into the water (Chorus and Bartram, 1999). For cyanobacteria-containing water, coagulation, flocculation and dissolved air flotation have been shown to be more effective than sedimentation (Falconer, 2005). A comparison of coagulation/flocculation/sedimentation with coagulation/flocculation/dissolved air flotation in the removal of *Microcystis* showed that the latter was a more efficient treatment process (Teixeira and Rosa, 2006).

Two types of membrane filtration, microfiltration and ultrafiltration, are commonly used to remove contaminants from drinking water. Both microfiltration and ultrafiltration as treatments or in replacing the conventional sand filtration have been shown to be effective in removal of intact cyanobacterial cells (Zhou and Smith, 2002; Gijsbertsen-Abrahamse *et al.*, 2006). An important point when considering filtration is the lysis of cells. Dissolved microcystins have been shown to be removed by some reverse osmosis and nanofiltration membranes, but the removal depends on the membrane pore size and water quality (Gijsbertsen-Abrahamse *et al.*, 2006).

The most common chemical oxidants used in drinking water treatment are ozone, hydroxyl radicals, chlorine, chlorine dioxide, chloramine and permanganate. Chlorination and ozonation are effective for the removal of microcystins (Nicholson *et al.*, 1994; Newcombe, 2002). Chlorination can be performed by using liquified chlorine gas, sodium hypochlorite solution or calcium hypochlorite granules and chlorine generators. Once cyanobacterial cells have been removed from water, the dissolved toxins are potentially susceptible to oxidation by disinfectants. The efficiency of chlorination seems to depend mainly on the chloride compounds and the concentration used.

Ozone can be used as a primary disinfectant. Ozone gas is formed by passing dry air or oxygen through a high-voltage electric field. Ozonation has been shown to be a very effective method for destroying microcystins and nodularins. In Europe and North America, ozonation has primarily been used for disinfection purposes or to remove colour and odour from water (Langlais *et al.*, 1991). Ozone was initially used at the beginning of water treatment mainly to inactivate viruses and bacteria. In recent years, many water treatment plants have included a two-stage ozonation treatment (Westrick, 2008; Miao *et al.*, 2010). Ozonation has previously been shown to rapidly achieve destruction of microcystins, nodularin and anatoxin-a (Keijola *et al.*, 1988; Himberg *et al.*, 1989). In the microcystin structure, ozone reacts with the conjugated double bond and single double bond in the Adda and the Mdha group, respectively (Onstad *et al.*, 2007).

Microcystins have been shown to be very stable under natural sunlight (Tsuji *et al.*, 1994), whereas ultraviolet light around the absorption maximum of microcystins is able to decompose

the toxins (Tsuji *et al.*, 1995). Ultraviolet light combined with hydrogen peroxide treatment has shown to be quite efficient in microcystin removal (Rositano and Nicholson, 1994).

Furthermore, biological treatment of water is a method used for cyanotoxin removal from drinking water. Biologically active filtration in the form of river bank filtration and both slow and rapid filtration have been reported to remove or to inactivate microcystins in drinking water (e.g. Lahti *et al.*, 2001; Bourne *et al.*, 2006) and are discussed more in detail in the following section.

### 1.8 Biological degradation of cyanobacterial toxins

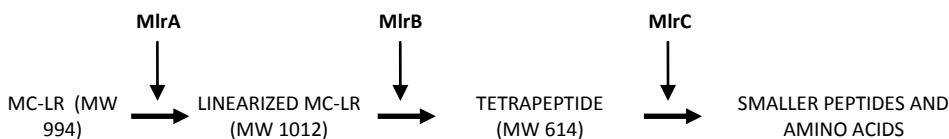
People are frequently exposed to cyanobacterial toxins as well as other microbial contaminants through drinking water. Therefore there is a need for simple, low-cost and effective water treatment procedures. Conventional water treatment procedures discussed in the previous section are in some cases insufficient in the removal of cyanobacterial toxins from drinking water, especially during cyanobacterial blooms. If the cyanobacterial cells are not removed by traditional water treatment methods, the cells and therefore the toxins remain in the drinking water and must be degraded to non-toxic compounds. Since microcystins have been released into the water body, the toxins can persist for weeks (Jones and Orr, 1994) before they are adequately degraded by for example bacteria.

Different biological methods have been applied to remove cyanobacteria and their toxins (reviewed in Chorus and Bartram, 1999). One type of these methods is the use of microorganisms or biofilms capable of degrading microcystins. Biological treatment for removal of toxin contaminants is becoming more useful as toxins can be removed without the addition of chemicals that may have the potential to produce undesirable by-products. Methods utilizing microcystin-degrading microorganisms can be classified into two groups. One is the use of biofilms grown on the surface of substrates within bioreactors, such as biological sand (Bourne *et al.*, 2006; Ho *et al.*, 2006 and 2007), biofilm-reactors based on immobilised microorganisms (Tsuji *et al.*, 2006), biological treatment facilities combined with conventional treatment processes (Saitou *et al.*, 2002), and granular activated carbon filters (Wang *et al.*, 2007). The other group depends on specific microorganisms efficient in microcystin-degradation, such as bacteria of the *Sphingomonas* sp. (Saito *et al.*, 2003; Valeria *et al.*, 2006) and *Sphingopyxis* sp. (Okano *et al.*, 2009).

Different variants of microcystins have been demonstrated to be degraded after incubation with water from a lake in Japan, which is frequently contaminated with cyanobacteria (Ishii and Abe, 2000). A more effective degradation was observed after adding bed sediment or mud from the lake, whereas no degradation could be observed after incubation with boiled water. Christoffersen *et al.* (2002) found out that bacteria can efficiently degrade microcystins in natural waters with previous cyanobacterial contamination and that the degradation process is rapid and without lag phase.

Many other studies have also reported biological degradation of microcystin in natural waters from lakes and reservoirs (Jones and Orr, 1994; Rapala *et al.*, 1994; Cousins *et al.*, 1996; Tsuji *et al.*, 1996). Several strains of the genus *Sphingomonas* have been reported to degrade microcystins (Jones *et al.*, 1994; Park *et al.*, 2001; Saitou *et al.*, 2003; Ishii *et al.*, 2004; Harada *et al.*, 2004; Valeria *et al.*, 2006). Seventeen strains of Gram-negative bacteria with the ability to degrade microcystins were isolated by Lahti *et al.* (1997). Table 1.3 lists strains reported to degrade microcystins in water, and so far these appear to mostly be limited to the *Sphingomonas* sp. Other reported microcystin-degrading bacteria include *Pseudomonas aeruginosa* (Takenaka and Watanabe, 1997), *Paucibacter toxinivorans* (Rapala *et al.*, 2005) and *Sphingosinicella microcystinivorans* (Maruyama *et al.*, 2006). Recently, Gram-positive bacteria isolated from freshwater and identified as *Arthrobacter* sp., *Brevibacterium* sp. and *Rhodococcus* sp. were shown to remove MC-LR (Manage *et al.*, 2009). The mechanism of MC-LR removal for *Rhodococcus* sp. C1 (Lawton *et al.*, 2011) was shown to be similar to the previously reported degradation pathway for *Sphingomonas* by Bourne *et al.* (1996).

Within the genome of the first isolated microcystin-degrading bacterium, *Sphingomonas* sp. ACM-3962, Bourne *et al.* (1996 and 2001) identified a gene cluster, *mlrA*, *mlrB*, *mlrC* and *mlrD*, responsible for the degradation of MC-LR. The authors determined that the *mlrA* gene encoded an enzyme responsible for the hydrolytic cleaving of the cyclic structure of MC-LR (ring-opening at the Adda-Arg peptide bond). The resulting linear MC-LR molecule was then sequentially hydrolysed by peptidases encoded by the *mlrB* and *mlrC* genes to a tetrapeptide and further to smaller peptides and amino acids (Fig. 1.4). The final gene, *mlrD*, encoded for a possible transporter protein that may have allowed for active transport of microcystin or its degradation products. Various studies have designed qualitative polymerase chain reaction assays for detection of *mlrA* (Saito *et al.*, 2003; Ho *et al.*, 2006 and 2007). Saito *et al.* (2003) reported gene homologues of *mlrA* in two microcystin-degrading bacteria, *Sphingomonas* sp. MD-1 and *Sphingomonas* sp. Y2, both of which were previously isolated from Japanese lakes. More recently, Hoefel *et al.* (2009) designed and optimised a quantitative real-time polymerase chain reaction assay for the detection of the *mlrA* gene.



**Figure 1.4.** MC-LR degradation pathway by *Sphingomonas* sp. ACM-3962; MlrA-C: microcystinases A-C (modified from Bourne *et al.*, 1996).

**Table 1.3.** Reported microcystin-degrading bacteria

Bacterial strain	Degradable toxins	Reference
<i>Sphingomonas</i> sp. MJ-PV	MC-LR	Jones <i>et al.</i> , 1994
<i>Pseudomonas aeruginosa</i>	MC-LR	Takenaka and Watanabe, 1997
17 different strains (Gram-negative, <i>Proteobacteria</i> )	MCs	Lahti <i>et al.</i> , 1998
<i>Sphingomonas</i> sp. ACM-3962	MC-LR, MC-RR	Jones and Orr 1994; Bourne <i>et al.</i> , 1996 and 2001
<i>Sphingomonas</i> sp. Y2	MC-LR, MC-RR, MC-YR, 6(Z)-Adda-MC-LR	Park <i>et al.</i> , 2001; Maruyama <i>et al.</i> , 2003 and 2006
<i>Sphingomonas</i> sp. MD-1	MC-LR, MC-RR, MC-YR	Saito <i>et al.</i> , 2003
<i>Sphingomona stygia</i>	MC-LR, MC-RR, MC-YR	Saitou <i>et al.</i> , 2003
<i>Sphingomonas</i> sp. B9	MC-LR, MC-RR, 3-dmMC-LR, dhMC-LR, MC-LR-Cys, NOD	Harada <i>et al.</i> , 2004, Imanishi <i>et al.</i> , 2005
<i>Sphingomonas</i> sp. 7CY	MC-LR, MC-RR, MC-LY, MC-LW, MC-LF	Ishii <i>et al.</i> , 2004
<i>Paucibacter toxinivorans</i> sp. nov.	MC-LR, MC-YR, NOD	Rapala <i>et al.</i> , 2005
<i>Sphingomonas</i> sp. MDB2	MCs	Maruyama <i>et al.</i> , 2006
<i>Sphingomonas</i> sp. MDB3	MCs	Maruyama <i>et al.</i> , 2006
<i>Sphingomonas</i> sp. CBA4	MC-RR	Valeria <i>et al.</i> , 2006
<i>Sphingomonas wittflariensis</i> LH21	MC-LR, MC-LA	Ho <i>et al.</i> , 2007
<i>Burkholderia</i> sp.	MC-LR, [D-Leu <sup>1</sup> ]MC-LR	Lemes <i>et al.</i> , 2008
<i>Sphingopyxis</i> sp. C-1	MC-LR	Okano <i>et al.</i> , 2009
<i>Brevibacterium</i> sp.	MC-LR	Manage <i>et al.</i> , 2009
<i>Arthrobacter</i> sp.	MC-LR	Manage <i>et al.</i> , 2009
<i>Rhodococcus</i> sp.	MC-LR	Manage <i>et al.</i> , 2009
<i>Methylobacillus</i> sp. strain J10	MC-LR, MC-RR	Hu <i>et al.</i> , 2009
<i>Stenotrophomonas</i> sp. strain EMS	MC-LR, MC-RR	Chen <i>et al.</i> , 2010
<i>Microbacterium</i> sp.	MC-LR	Ramani <i>et al.</i> , 2011
<i>Rhizobium gallicum</i>	MC-LR	Ramani <i>et al.</i> , 2011

### 1.9 The gut and human intestinal microbiota

The human gastrointestinal microbiota is a complex community. Within the gastrointestinal tract, different habitats such as mouth, stomach, small intestine (especially lower jejunum and ileum), large intestine (caecum, colon) and rectum all harbour bacteria. The fully developed healthy human microbiota is a multifaceted ecosystem, with more than 1000 different bacterial species present. The microorganisms constituting the microbiota are unevenly distributed along the digestive tract; the stomach and duodenum having less than  $10^3$  bacterial cells per gram of intestinal contents, ileus and distal ileum  $10^2$ – $10^3$  cells/g and the large intestine  $10^{10}$ – $10^{12}$  cells/g being the location of most microbiota activities, while the predominant genera changes from Gram-positive aerobes to Gram-negative anaerobes (reviewed in e.g. Patel and Lin, 2010). The species appearing in the different parts are selected on the basis of the environmental conditions present, such as acidity and the availability of nutrients and oxygen.

Bifidobacteria form a minor part in the human adult microbiota, even though they form the predominant genus of the neonate microbiota (Conway, 1995). It is therefore believed that by adding these bacteria as probiotics to the diet, the normal microbiota can be altered. This alteration might then prevent the adhesion of pathogenic organisms, modulate bacterial enzyme activity and influence the gut mucosal permeability (Salminen *et al.*, 1996).

The main site of action for the health benefits of probiotic bacteria is the gut. The intestinal mucosa forms a barrier between the external and internal environment of the human body. The gastrointestinal tract represents a complex ecosystem in which a balance exists between the intestinal microbiota and the host. It has been estimated that bacteria account for 35–50% of the volume content of the human colon (Vanhoutte *et al.*, 2004; Ley *et al.*, 2006). The microbiota mainly consists of facultative anaerobes and obligate anaerobes. Approximately 95% of the intestinal bacterial population in humans is comprised of obligate anaerobes, including *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus* and *Bacteroides*. 1–10% of the intestinal population is comprised of facultative anaerobes, including *Lactobacillus*, *Escherichia coli*, *Klebsiella*, *Streptococcus*, *Staphylococcus* and *Bacillus* (Guarner and Malagelada, 2003).

The intestinal microbiota can be identified as an active “organ” with the gastro-intestinal tract serving as a large mucosal surface that bridges the gap between inside outside the body. Along this mucosal interface, microbes and foreign antigens colonizing or passing through the gastro-intestinal tract interact with important components of the immune system. The microbiota is involved in different processes, such as the improvement of nutrient bioavailability and degradation of non-digestible dietary compounds, the supply of new nutrients, and the removal of harmful, toxic and non-nutritional compounds. The main positive functions of the microbiota on the host organism are the following: participation in the defence system of the intestinal mucosa, resistance to colonisation of pathogens (Cummings and Macfarlane, 1997), production of short chain fatty acids, metabolites that play important physiological functions in fermentation (Resta, 2009), production of vitamins, especially vitamins B and K (Lanning *et al.*, 2005), interactions with the mucosal immune system (Tsuji *et al.*, 2008), and degradation of xenobiotics (Reiff *et al.*, 2009). Alteration of the microbiota of the intestine, such as during antibiotic use, disease and aging, can negatively affect the beneficial role of the microbiota.

### 1.10 Probiotic bacteria

Elie Metchnikoff, the Nobel Prize winner for the discovery of phagocytosis, proposed a scientific basis for the beneficial effects of bacteria in yoghurt at the start of the 20<sup>th</sup> century by attributing the long life of Bulgarian peasants to their consumption of fermented milk containing lactic acid bacteria (Metchnikoff, 1907). He suggested that usage of fermented milk would decrease toxic microbial activity in the colon.



Probiotics were earlier defined by Fuller (1991) as “live microbial food supplements which beneficially affect the host either directly or indirectly by improving its intestinal microbial balance”. Today, the most commonly accepted definition states that probiotics are “live microbial food supplements which, when given in adequate amounts have a demonstrated beneficial effect on human health” (WHO, 2002). In order to be effective the probiotic micro-organisms must be able to survive the digestive conditions, including bile acids, and they must be able to colonise the gastrointestinal tract at least temporarily without any harm to the host (FAO/WHO, 2002). Only certain strains of micro-organisms have these properties. Most probiotic micro-organisms are members of two bacterial genera, *Lactobacillus* and *Bifidobacterium* (Saxelin *et al.*, 2005), but they can also belong to yeasts, especially *Saccharomyces*, or enterococci (Shortt, 1999). In probiotic foods the number of these beneficial bacteria is increased and maintained at a high level. Among the most common probiotic microbes are strains of *Lactobacillus (L.) acidophilus* and *Bifidobacterium (B.) animalis* subsp. *lactis*.

There are several criteria for a probiotic bacterium, including being able to exert proven beneficial effects on the host, being non-pathogenic and non-toxic, being present as living cells, being able to survive the passage through the gut and resistance against metabolic enzymes and being stable and remain viable through storage (Pathmakanthan *et al.*, 2000). Many probiotic organisms originate in fermented foods, and they have a long history of safe use in human consumption. The International Dairy Federation has prepared a list of safe microorganisms with a documented history of use in food (IDF, 2002). The list was recently updated and reflects advances in fermented food, microbial culture technology and knowledge, including probiotics (IDF, 2011). Today the most important classification systems are the Qualified Presumption of Safety (QPS) in Europe (EFSA, 2010) and the generally recognised as safe (GRAS) status and notification system in the United States (Food and Drug Administration, U.S.). These systems largely define which bacteria can be used as safe probiotics.

When discussing probiotic bacteria, the term lactic acid bacterium is often used. Lactic acid bacteria are a heterogeneous group of Gram-positive, non-spore-forming cocci or rods, which produce lactic acid as the major end-product of carbohydrate fermentation. Lactic acid bacteria comprise strains from the genera *Aerococcus*, *Alliococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Masood *et al.*, 2011). Lactic acid bacteria used as probiotics only include strains from the genus *Lactobacillus* (Table 1.4).

The genus *Bifidobacterium* is often considered in the same context as lactic acid bacteria, but is phylogenetically unrelated to them. Bifidobacteria have a unique mode of sugar fermentation. The genus *Propionibacterium* is of special interest in food production with several strains being used as dairy starters to produce flavour compounds, carbon dioxide and propionic acid, a preserving agent (Ouweland, 2004). In recent studies *Propionibacterium freudenreichii* has also

been shown to have probiotic properties (Ouweland, 2004). Furthermore, some other bacteria, yeast and moulds are described as probiotics (Goldin, 1998).

There are several important modes of action for probiotic bacteria, including modification of gut pH, colonisation ability, inhibition of the colonisation, adhesion and invasion of pathogens, direct antimicrobial effect, replacement of already adhered pathogens, competing for available nutrients and growth factors, regulation of the immune system of the host, normalisation of the gut microbiota, and different metabolic effects (reviewed in Salminen *et al.*, 1998; Andersson *et al.*, 2001). It is essential that the probiotic bacteria retain viability during processing and transit through the gastrointestinal tract. The beneficial effects of consumption of probiotic bacteria include improving intestinal tract health, enhancing the immune system, and synthesizing and enhancing the bioavailability of nutrients. Probiotics have well-documented health effects in the alleviation of symptoms of lactose intolerance, reduction in the risk of allergies and acute gastroenteritis (rotavirus, antibiotic-associated and travellers' diarrhoea), and improving bowel regularity at times of constipation and inflammatory bowel disorders (Crohn's disease and ulcerative colitis) (Fooks and Gibson, 2002; Tuohy *et al.*, 2003; Saxelin *et al.*, 2005; Sazawal *et al.*, 2006) (discussed in section 1.11).

### 1.10.1 Lactobacilli

Lactobacilli are normal inhabitants of the human intestine and vagina. Lactobacilli are Gram-positive facultative anaerobes. They are non-spore forming and non-flagellated rod or coccobacilli. They are either aerotolerant or anaerobic and strictly fermentative. In strictly fermentative bacteria, glucose is fermented predominantly to lactic acid. Lactobacilli are classified as lactic acid bacteria. Lactobacilli used as probiotics include *L. acidophilus*, *L. brevis*, *L. bulgaricus*, *L. casei*, *L. cellobiosus*, *L. crispatus*, *L. curvatus*, *L. fermentum*, *L. gasseri*, *L. johnsonii*, *L. plantarum*, *L. rhamnosus* and *L. salivarius*. Lactobacilli common in the food industry belong to the European QPS status organisms, which can be used in foods and feeds.

*L. plantarum* strains used in some studies of this thesis have been isolated from traditionally fermented dadih. 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 and capped with banana leaves (Akuzawa and Surono, 2002). Different bacteria, mainly lactobacilli (*L. plantarum*, *L. casei*, and others), enterococci (*E. faecium*), and some pediococci can be found in the fermented dadih product (Surono and Hosono, 1996; Surono, 2003). *L. plantarum* is a versatile species present in a range of environmental niches including dairy, meat, and vegetable fermentations (de Vries *et al.*, 2006). Studies on potential probiotic properties of indigenous lactic acid bacteria isolated from dadih have demonstrated antimutagenic, mutagen-binding, and cholesterol-binding properties, as well as acid and bile tolerance, good adhesion properties, and antipathogenic properties (Surono, 2003; Pato *et al.*, 2004; Dharmawan *et al.*, 2006; Collado *et al.*, 2007a and 2007b).

### 1.10.2 Bifidobacteria

Bifidobacteria are normal inhabitants of the human and animal colon. Newborns, especially breast-fed, are colonised with bifidobacteria within days after birth (Fuller, 1991). Bifidobacteria were first isolated from the faeces of breast-fed infants. In general, bifidobacteria form the hallmark of the microbiota of a healthy breastfed infant, and bifidobacteria may constitute over half of the intestinal bacteria at this age. The population of these bacteria in the colon appears to be relatively stable until advanced age when it appears to decline (Gueimonde *et al.*, 2010). The bifidobacteria population is influenced by a number of factors, including diet, antibiotics and stress (Turrioni *et al.*, 2011). Bifidobacteria are Gram-positive anaerobes. They are non-motile, non-spore forming and catalase-negative. They are saccharolytic organisms that produce acetic acid and lactic acid without generation of CO<sub>2</sub>, except during degradation of gluconate. Bifidobacteria used as probiotics include *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. longum*, and *B. thermophilum* (Goldin, 1998; Salminen *et al.*, 2004).

**Table 1.4.** Examples of bacterial species used as probiotics

<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Enterococcus</i>	Other bacteria
<i>L. acidophilus</i>	<i>B. adolescentis</i>	<i>E. faecalis</i>	<i>Bacillus cereus</i>
<i>L. casei</i>	<i>B. animalis</i> ( <i>B. lactis</i> )	<i>E. faecium</i>	<i>Bacillus subtilis</i>
<i>L. fermentum</i>	<i>B. bifidum</i>		<i>Escherichia coli</i>
<i>L. gasseri</i>	<i>B. breve</i>		<i>Clostridium butyricum</i>
<i>L. johnsonii</i>	<i>B. longum</i>		<i>Lactococcus lactis</i>
<i>L. paracasei</i>	<i>B. thermophilum</i>		<i>Propionibacterium freudenreichii</i> ssp.
<i>L. plantarum</i>			<i>Shermanii</i>
<i>L. reuteri</i>			<i>Saccharomyces cerevisiae</i>
<i>L. rhamnosus</i>			<i>Streptococcus salivarius</i>
<i>L. salivarius</i>			<i>Streptococcus thermophilus</i>

based on Donohue and Salminen, 1996; Shortt, 1999

### 1.10.3 Functional foods

Functional foods have become an important and rapidly expanding area of the food development by promoting the health benefits provided by functional ingredients in the food products. Functional foods are targeted to improve the balance and activity of the gastrointestinal tract, specific targets including gastrointestinal functions, such as bowel habits, mucosal motility and modulating epithelial cell proliferation. Furthermore, functional foods target gastrointestinal functions that are associated with a balanced colonic microbiota, control of nutrient bioavailability, modification of gastrointestinal immune activity, and functions that are mediated by the endocrine activity of the gastrointestinal system. A functional food can be defined as a food containing a component that affects one or a limited number of functions in the body, which have positive effects on health, or if it has a physiological or psychological effect beyond the traditional nutritional effect (Bellisle *et al.*, 1998). The European Union has set up regulations for

foods with approved health claims (European Union regulation on nutrition and health claims, 2006), in which the same rules for the use of such claims in advertisements, labelling and presentation of foods, including food supplements, are implemented throughout the European Union.

Probiotics and prebiotics are available commercially in many forms, such as foods, dietary supplements, and clinical therapeutic agents with oral or non-oral delivery. At present, probiotics are extensively consumed as fermented dairy products, such as yogurt or freeze-dried cultures. To be effective, probiotics must be capable of being prepared in an economically viable manner and on large scale, and during use and storage the probiotic should remain viable and stable. Maintaining the viability, stability, and functionality of probiotics during processing, formulation and storage is important for delivery of the health benefits (Saarela *et al.*, 2002). An important area of research today is enhancing the stability of probiotics in industrial processes and keeping the probiotic properties intact during growth, harvest and storage (Tuomola *et al.*, 2001; Grześkowiak *et al.*, 2011).

#### **1.10.4 Selection criteria for probiotics**

The guidelines for the selection of probiotic strains for food use have been established by the FAO and the WHO expert consultation group (FAO/WHO, 2002). According to the guidelines the probiotic strain should be identified by genotypic and phenotypic methods as current knowledge suggests that probiotic effects are strain specific. Thereafter, functional characterisation, efficacy and safety tests should be performed with *in vitro* assays and, when appropriate, in animal studies. Most commonly used *in vitro* tests include ability to reduce pathogen adhesion to surfaces, resistance to gastric acid and bile acid, persistence in the gastrointestinal tract and antimicrobial activity against potentially pathogenic bacteria (Dunne *et al.*, 1999; FAO/WHO, 2002). Next, new probiotic strains are tested by using standard methods in two clinical evaluations; phase 1 (safety assessment) and phase 2 (efficacy assessment) studies. The safety assessment does not concern strains already on the IDF safety list (IDF, 2002). It is also recommended that human trials are repeated by more than one centre in order to confirm the results. Finally, the guidelines also provide recommendations for the labelling of probiotic food products, such as contents, numbers of viable bacteria, end of shelf-life and proper storage conditions.

In general, for microorganisms to be considered as probiotics, they should be isolated from the same species as the intended host; have a demonstrable beneficial effect on the host; be non-pathogenic, non-toxic, and free of significant adverse side effects; be able to survive through the gastrointestinal tract; be stable during the intended product shelf life and contain an adequate number of viable cells to confer the health benefit; be compatible with product format to maintain desired sensory properties; and labelled accurately (reviewed in Collado *et al.*, 2009).

### 1.10.5 Safety assessment of probiotics

The use of lactic acid bacteria and bifidobacteria as probiotics requires a safety assessment. The functional properties of the strains should be well studied and documented. Probiotics belonging to the genera *Lactobacillus* and *Bifidobacterium* have a good safety status as they are present in the normal intestinal microbiota of humans and have a long history of safe use in fermented foods (Tuohy *et al.*, 2003). Species from other bacterial genera such as *Streptococcus*, *Bacillus*, and *Enterococcus* have also been used as probiotics, but there are concerns surrounding the safety of such probiotics because these genera also contain pathogenic species. Enterococci can be associated with nosocomial infections and may have antibiotic resistance (Mundy *et al.*, 2000). Infections caused by probiotic bacteria other than enterococci are quite rare and usually originate from the body's indigenous microbiota rather than from ingested food. The patients have often had a reduced immune function or underlying diseases (Adams, 1999). Boyle *et al.* have reviewed the safety of probiotics and discussed areas of uncertainty regarding their use, including reported cases of sepsis related to probiotic use (2006).

Proper taxonomic identification of the probiotic is the starting point for safety as well as efficacy assessment (FAO/WHO, 2002). When considering the safety of probiotics, the side effects that theoretically could occur include systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer (Marteau, 2001). Probiotics should be characterised by determining at least the infectivity in immunocompromised animal models, harmful metabolic activity, toxin production, hemolytic activity, antibiotic resistance, side effects in humans and adverse incidents in consumers (FAO/WHO, 2002). In Europe, the safety assessment is based on the EFSA QPS-system, which continuously evaluates the safety data on microbes added into foods and feeds (EFSA, 2010).

### 1.11 Beneficial health effects of probiotic bacteria

The literature available on the potential of probiotic bacteria in the treatment of different infections and disorders and on proven health effects is extensive. Only some of the reported beneficial health effects are presented in this section.

Probiotics have been linked with a range of beneficial effects on human health, both well-documented and less studied suggested health effects. It should therefore be remembered that there is a major difference between a proposed and a well-established health effect. Probiotics have well-documented health effects in the alleviation of symptoms of lactose intolerance, reduction in the risk of allergies and acute gastro-enteritis (rotavirus, antibiotic-associated and travellers' diarrhoea), constipation, inflammatory bowel disorders (Crohn's disease and ulcerative colitis), and necrotizing enterocolitis (reviewed in Ouwehand *et al.*, 2002; Parvez *et al.*, 2006; Masood *et al.*, 2011). Several proposed health effects of probiotic bacteria have also been reported, including adjuvant therapy in *Helicobacter pylori* eradication (Tong *et al.*, 2007),

enhancement of the adaptive immune response, treatment or prevention of urogenital and respiratory tract infections, and prevention or alleviation of allergies and atopic diseases in infants (Tuohy *et al.*, 2003; Saxelin *et al.*, 2005). Positive results on the effects of probiotics on the respiratory system have also been obtained, especially in preventing and reducing the severity of respiratory infections (Perdigon *et al.*, 1999).

Generally, health benefits of probiotics have often been characterised using single bacterial strains, and therefore the proven effects can only be defined for the specific strain studied (Salminen *et al.*, 2004). One of the most studied clinical uses of probiotics in humans is in the management of diarrhoeal diseases (reviewed recently in Hickson, 2011). This group of diseases includes antibiotic-associated diarrhoea and infective diarrhoea, such as rotavirus diarrhoea (Casas and Dobrogosz, 2000), or traveller's diarrhoea in both adults and children (Pathmakanthan *et al.*, 2000; Boyle *et al.*, 2006; Allen *et al.*, 2010). Several Cochrane-reviews have also analysed and reported the effect of probiotics in diarrhoeal diseases (e.g. Johnston *et al.*, 2007; Allen *et al.*, 2010). The beneficial effect of probiotics on rotavirus infection is explained by the stabilizing effect of probiotics on the microbiota and the reduction of increased gut permeability caused by the infection (Isolauri, 2001). Potentially also inactivation or binding of viruses maybe involved (Salminen *et al.*, 2010). Antibiotic-associated diarrhoea results from an imbalance in the colonic microbiota caused by antibiotic therapy. Microbiota alteration changes carbohydrate metabolism with decreased absorption of short-chain fatty acids and an osmotic diarrhoea as a result. Another consequence is overgrowth of potentially pathogenic organisms, such as *Clostridium difficile* (Parkes *et al.*, 2009). The Cochrane Group produced a systematic review (Pillai and Nelson, 2008) to evaluate the evidence for the treatment of *Clostridium difficile*-associated diarrhoea. The authors concluded that there was insufficient evidence to support the use of probiotic therapy as an addition to antibiotic therapy, and no evidence for the use of probiotics alone, in the treatment of *Clostridium difficile* colitis.

Clinical intervention studies have shown that specific probiotic strains may also be able to shorten the duration or reduce the risk of certain viral infections. One example is the shortening the duration of viral diarrhoea, and reducing the risk of viral diarrhoea especially in infants and children (Isolauri *et al.*, 1991). Moreover, selected probiotics have been effective also against respiratory tract infections of viral origin (Hatakka *et al.*, 2001; Cobo Sanz *et al.*, 2006).

Probiotic treatment might alleviate the side effects of antibiotics and reduce the incidence and severity of antibiotic-associated diarrhoea as indicated in several reports (D'Souza *et al.*, 2002; Sazawal *et al.*, 2006; Szajewska *et al.*, 2006). For example, treatment with probiotics including *L. rhamnosus* may reduce the risk of antibiotic-associated diarrhoea, improve stool consistency during antibiotic therapy, and enhance the immune response after vaccination (D'Souza *et al.*, 2002). Probiotics appear to be most effective in the prevention and treatment of rotavirus and antibiotic-associated diarrhoea, while the effect on travellers' diarrhoea needs further assessment.

The effect of probiotic bacteria to alleviate symptoms of lactose intolerance and of food allergies in infants (reviewed in Salminen *et al.*, 2004; Aureli *et al.*, 2011; Masood *et al.*, 2011) is well established. Several studies have focused on immunomodulation by probiotic treatment, and many potential benefits are discussed, such as stimulation of non-specific intestinal immune reactions with bifidobacteria (Casas and Dobrogosz, 2000) and prevention of the development of atopic disease (Kalliomäki *et al.*, 2001). The role of probiotics in the recurrence of allergic respiratory symptoms in children was recently studied by assessing whether a daily, long-term (12 months) consumption of a probiotic containing fermented milk could improve and modify the immunological profile of children with allergic symptoms (Giovannini *et al.*, 2007). The study showed that probiotics supplementation reduced the recurrence of yearly rhinitis and lowered the incidence of allergic rhinitis in the children.

The potential of probiotics to decrease serum cholesterol has been investigated. It has been concluded that evidence of hypocholesterolemic effects is available *in vitro* and *in vivo* in animals, possibly via deconjugating bile and increasing faecal excretion of bile acids (Casas and Dobrogosz, 2000; Lichtenstein and Goldin, 2004). Animal studies have demonstrated the efficacy of a range of probiotic bacteria to be able to lower serum cholesterol levels (Sanders, 2000). Human studies have showed conflicting results, some studies indicating that probiotic bacteria may reduce serum LDL-cholesterol levels (reviewed in Ooi and Liong, 2010), while in other studies no such effect has been observed (de Roos *et al.*, 1999).

Probiotic supplementation has also been shown to be effective for alleviation of lactose intolerance symptoms in adults. As lactic acid bacteria actively convert lactose into lactic acid, ingestion of specific strains with high lactase activity may help lactose intolerant individuals tolerate dairy products (Sanders, 2000). Not all probiotic strains are able to ferment lactose or alleviate lactose intolerance, and lactase production is also strain dependent.

*Helicobacter pylori* is a Gram-negative spiral-shaped, micro-aerophilic rod colonizing the human gastric mucosa and causing peptic ulcers. Probiotic bacteria may affect *Helicobacter pylori* infections by eradication of the infection when used in combination with standard medical treatments. At the moment there is no standard in medical practice or regulatory approval for such treatment (Hamilton-Miller, 2003; Myllyluoma *et al.*, 2005).

Irritable bowel syndrome (IBS) is characterised by abdominal pain, bloating, and change in bowel habit, with an absence of any clear mucosal abnormality. Probiotics may be of benefit in managing the symptoms of IBS via a number of mechanisms, such as increasing mucosal interleukins and reducing pro-inflammatory cytokines. Different studies in adults have showed that *B. infantis*, *L. rhamnosus* GG, and a mixture of probiotics (*L. rhamnosus* GG, *L. rhamnosus* LC-705, *B. breve* Bb99 and *Propionibacterium freudenreichii* JS) can be effective in alleviating symptoms of IBS (Kajander *et al.*, 2005 and 2008).

Inflammatory bowel disease (IBD) is a chronic and recurrent inflammation generally affecting the colon or the small intestine and includes ulcerative colitis, pouchitis, and Crohn's disease. The use of probiotics in the treatment of IBD has been assessed (Gionchetti *et al.*, 2002; Marteau, 2006). The best results so far have been obtained on ulcerative colitis and chronic pouchitis (Gionchetti *et al.*, 2002).

Many studies have been aimed at explaining the mechanisms through which probiotics beneficially affect human health. Strengthening of the gut mucosal barrier could be one of the mechanisms involved. A large part of the microorganisms in the human gut are non-culturable using current methodology. However, these bacteria are active in the gut demonstrating that viability is not always a requirement for such health benefits; also non-viable probiotics have been demonstrated to have health benefits. Adhesion abilities of beneficial bacteria have been widely studied (Tuomola *et al.*, 2000). Adhesion to the intestinal mucosa, followed by at least transient colonization is considered important for probiotic bacteria to exert their favourable effects as it prolongs the contact period with the host, thus allowing more time for the probiotics to exert their beneficial health effects. Even among different lactobacilli, adhesion properties vary, and different possible binding sites including proteins, carbohydrates, or a combination of both are suggested in the literature (Rojas and Conway, 1996; Alander *et al.*, 1999; Tuomola *et al.*, 2000; Rojas *et al.*, 2002). Current data support the hypothesis that the use of probiotic combinations selected for specific targets may have a major impact on human health (Collado *et al.*, 2007c; Collado *et al.*, 2008).

### **1.12 Removal of different contaminants by probiotic bacteria**

Previous studies have shown the effect of probiotic bacteria in the removal of different contaminants, such as heavy metals and mycotoxins. Also the removal of Shiga toxin has been assessed. The most efficient microbial species and strains in the removal of these compounds vary between contaminants tested. In a pilot study investigating the removal of cyanotoxins (Meriluoto *et al.*, 2005) it was reported that three probiotic strains, *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. lactis* Bb12, were able to remove MC-LR from solution. The highest removal in that study was observed with heat-treated bacteria, but further investigation (shown in the result part of this thesis) concluded that heat-treated bacteria were in fact inefficient in microcystin-removal.

Possible ways of interaction of probiotics with different contaminants, such as toxins and viruses, are illustrated in Fig. 1.5. Some of the studies and the characterisation of probiotics and their specific interactions with potentially pathogenic bacteria, bacterial toxins, fungal toxins and heavy metals are described in this section.

#### **1.12.1 Mycotoxins**

Previous studies have shown that specific strains of probiotic bacteria are able to bind the mycotoxins ochratoxin A (Turbic *et al.*, 2002) and aflatoxins B1 (AFB<sub>1</sub>) (El-Nezami *et al.*, 1998a;



Peltonen *et al.*, 2001) and M1 (Pierides *et al.*, 2000). Many of these studies have involved *Lactobacillus* strains, and physical binding has been proposed as one mechanism of toxin removal. Acid and heat treatment of the probiotics had varying effects on binding properties, but often caused increased toxin binding (Haskard *et al.*, 2000 and 2001).

Several bacterial strains have been tested for their ability to bind aflatoxins and other mycotoxins to their surface. Five strains of probiotic *Lactobacillus* and one *Propionibacterium* were shown to be more efficient in aflatoxin-removal than *Escherichia coli* (El-Nezami *et al.*, 1998a). Among the five strains, *L. rhamnosus* strains GG and LC-705 appeared to be most efficient in AFB<sub>1</sub>-removal, removing approximately 80% of AFB<sub>1</sub> within minutes of incubation. These two strains were later confirmed as the most efficient strains in AFB<sub>1</sub>-removal among nine strains of *Lactobacillus* (Haskard *et al.*, 2001). Peltonen *et al.* (2001) also studied a range of lactobacilli and bifidobacteria and found differences in AFB<sub>1</sub>-binding abilities between the studied strains. Also the bacterial concentration was shown to influence the AFB<sub>1</sub>-removal.

Chemical and physical treatments of the probiotic bacteria affected their AFB<sub>1</sub>-removal ability. Heat and acid treatment enhanced AFB<sub>1</sub>-binding by *L. rhamnosus* GG and LC-705 (El-Nezami *et al.*, 1998a and 1998b). In another study, viable strains of bifidobacteria were shown to bind 4–56% while heat-treated bacteria bound 12–82% of the AFB<sub>1</sub> (Peltonen *et al.*, 2001). As heat-treated bacteria were more efficient in AFB<sub>1</sub>-removal than viable cells, metabolic degradation was not considered the mechanism responsible for AFB<sub>1</sub>-removal. It was therefore suggested that the toxin is bound to the bacterial surface.

Besides *in vitro* studies the binding ability of three probiotic strains was tested *ex vivo* in the intestinal lumen of chicks (El-Nezami *et al.*, 2000). The study reported that strains removed 36–54% of the AFB<sub>1</sub> from the soluble fraction of the luminal fluid within one minute. These results imply that bacterial AFB<sub>1</sub> binding also appears under physiological conditions in the animal.

Also two *Fusarium* mycotoxins, zearalenone and its derivative  $\alpha$ -zearalenol have been shown to be removed by strains of *Lactobacillus* (El-Nezami *et al.*, 2002) similarly to the removal of AFB<sub>1</sub>.

### 1.12.2 Shiga toxin

The anti-infectious activity of probiotic bifidobacteria against Shiga toxin-producing *Escherichia coli* has been examined in a fatal mouse infection model. Specific strains of bifidobacteria were shown to have an anti-infectious activity against Shiga toxin in the mouse model (Asahara *et al.*, 2004). The important features of the mechanism responsible for the anti-infectious activity of bifidobacteria were inhibition of Shiga toxin production, while bacterial growth was not affected, and improvement of intestinal environmental factors, such as pH and acetate concentration. The pathogenicity observed in the infected controls was noticeably inhibited in the group colonised by bifidobacteria. A strain of *B. breve* was shown to have the anti-infectious activity, while strains of *B. bifidum* and *B. catenulatum* were inefficient (Asahara *et al.*, 2004).

### 1.12.3 Heavy metals

Cadmium and lead are highly toxic heavy metals. Unlike many other toxic compounds, they are not degradable and tend to accumulate in exposed organisms. The removal process of heavy metals, such as cadmium, lead and arsenic, has been studied in different strains of probiotic bacteria (Halttunen *et al.*, 2007a and 2007b). In addition, the possible mechanisms involved in the removal of cadmium and lead by lactic acid bacteria from water was investigated. Also the effect of other metals, reversibility of binding and recyclability of the biomass was assessed.

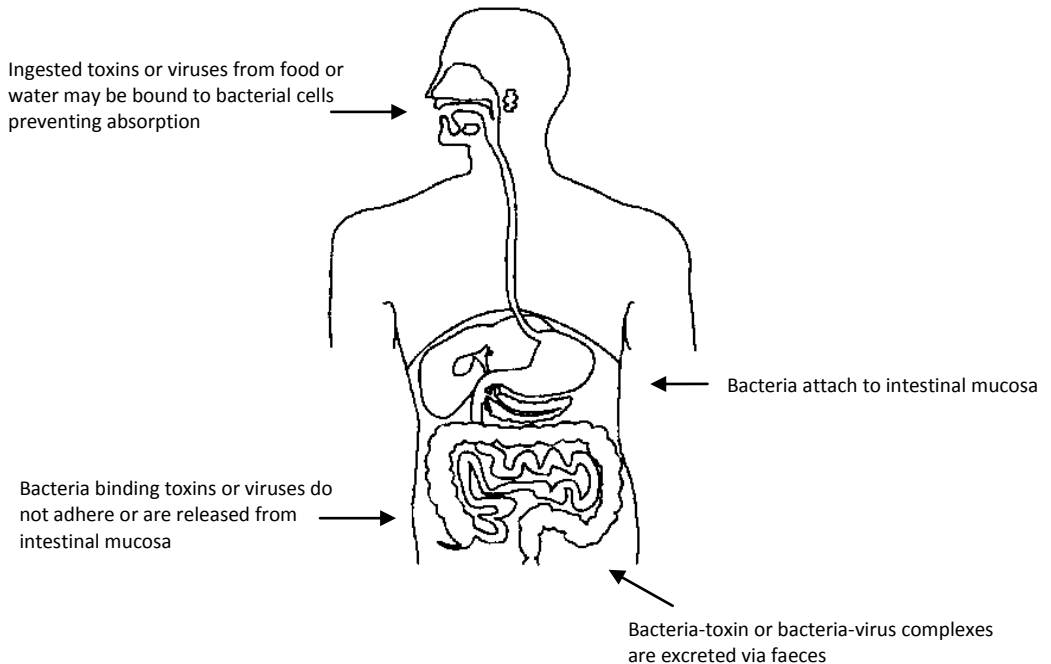
Specific lactic acid bacteria were observed to have a strain-specific capacity to bind cadmium and lead from water. Based on the maximum binding capacity, the most efficient strains for cadmium and lead removal were *B. longum* 46 and *L. fermentum* ME3 (Halttunen *et al.*, 2007a). Up to 99% of cadmium and 97% of lead were removed from solutions with initial metal concentrations of 100 and 1000 µg/L, respectively, within minutes of incubation. In addition, the removal of arsenic was studied. After chemical modification, *L. casei* DSM20011 was shown to remove anionic arsenic (Halttunen *et al.*, 2007b). The removal of arsenic, cadmium and lead was a rapid, pH-dependent and reversible surface process. Ion exchange was reported to be among the mechanisms behind cadmium and lead binding by lactic acid bacteria (Halttunen *et al.*, 2007a). This conclusion was supported by the reduced cadmium and lead removal in the presence of other cationic metals. Efficient desorption of cadmium and lead from the bacterial cells was achieved, although often more than one wash was needed to reach full desorption.

### 1.12.4 Probiotic pathogen interactions

The protective role of probiotic bacteria against gastrointestinal pathogens as well as the mechanisms responsible have been studied, as such interactions have been a criterion for selecting new probiotics for human use. The mechanisms by which probiotics exert their beneficial effects on the host are largely unknown. Among the identified mechanisms are the reduction of luminal pH, competition with pathogens for adhesion sites and nutritional sources, secretion of antimicrobial substances, toxin inactivation, and immune stimulation (Fooks *et al.*, 1999; Servin, 2004). Host-bacteria interaction mechanisms include physical bacteria-epithelium interaction, such as adhesion to mucosal and epithelial cells, stimulation of mucus secretion, production of defensive molecules, and support of gut barrier function. The bacteria-immune system interaction includes modulation and regulation of immune responses. Mechanisms of interactions between bacteria are exclusion and inhibition of pathogens by prevention of adhesion, secretion of antimicrobial substances, competition for nutrients and anti-toxin effects (Collado *et al.*, 2009).

Several studies suggest that combinations of specific probiotics strains may prove more effective than a single probiotic strain depending on the specific target. Combinations of known probiotic strains, such as *L. rhamnosus* GG and *B. lactis* Bb12, may have an improved impact on the

pathogen adhesion inhibition (Ouwehand *et al.*, 2000; Collado *et al.*, 2007d). Probiotic strains and their combinations were able to inhibit significantly the adhesion of *Bacteroides vulgatus*, *Clostridium difficile*, *Staphylococcus aureus* and *Cronobacter sakazakii* (Collado *et al.*, 2007d). Specific probiotic combinations were demonstrated to be more efficient in the inhibition of pathogen adhesion and they were able to enhance the inhibition percentages compared to when the probiotic strains were tested alone.



**Figure 1.5.** Interaction of probiotics with toxins and viruses in the human gut (modified from Salminen *et al.*, 2010).

## **2. AIMS OF THE STUDY**

The aims of this research work have been to characterise the potential of probiotic lactic acid bacteria and bifidobacteria in removal of the cyanobacterial peptide toxins microcystins and cytotoxin cylindrospermopsin from aqueous solutions. Different physiological conditions potentially affecting the removal efficiency were tested. These included the effect of pH, temperature, toxin concentration, bacterial cell density and cell viability (publications I and II). The cell viability and the impact of glucose as a source of energy were then studied more in detail (publication III). Furthermore, the location and mechanism of microcystin removal was investigated by studying a possible extracellular enzymatic degradation of microcystins and the degradation pathway was compared to other microcystin-degrading bacteria (publication IV).

Consequently, the specific aims of this study were to:

- Characterise the capacity of specific strains of probiotic lactobacilli and bifidobacteria to remove different cyanobacterial toxins from aqueous solutions and choose a number of specific strains optimal for cyanotoxin removal (I)
- Evaluate the potential of specific probiotic bacteria for decontamination of cyanotoxin-containing water (II)
- Assess factors and different conditions optimal for toxin removal (I–III)
- Investigate the nature of the removal process (III, IV)
- Investigate the hypothesis of extracellular enzymatic degradation of microcystins by probiotic bacteria (IV)
- Compare the degradation pathway of previously identified microcystin-degrading bacteria with the probiotic strains to identify the possible toxin-removal mechanism of probiotic bacteria (IV)

### 3. EXPERIMENTAL PROCEDURES

#### 3.1 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), trifluoroacetic acid (TFA) was from Fluka (Buchs, Switzerland) and hydrochloric acid (HCl) was from J.T. Baker (Deventer, the Netherlands). Water was purified to 18.2 MΩcm on a Milli-Q Synthesis system (Millipore, Molsheim, France). MC-LR test solutions were prepared in 0.01 M phosphate-buffered saline (PBS; pH 7) (chemicals from Merck, Darmstadt, Germany) and glucose was from J.T. Baker (Deventer, The Netherlands). Scintillation liquid OptiPhase HiSafe 3 was from PerkinElmer (USA). Ethylenediamine tetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), iodoacetic acid and a protease inhibitor cocktail (containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, and sodium EDTA) were obtained from Sigma-Aldrich (St. Louis, MO), and Suc-Ala-Ala-Phe-AMC from Bachem (Torrance, CA).

#### 3.2 Cyanobacterial cultures

*Microcystis* PCC 7820 (Institut Pasteur, Paris, France), *Anabaena* sp. 90 (originating from the culture collection of Prof. Kaarina Sivonen, University of Helsinki, Finland), *Anabaena lapponica* 966 (Spoof *et al.*, 2006) and *Microcystis* NIES-107 (National Institute for Environmental Studies, Tsukuba, Japan) were cultured in our laboratory. The strains were cultured in modified Z8 medium (Meriluoto and Spoof, 2005c). In the medium for the *Anabaena* strains nitrogen-containing compounds were omitted. The cyanobacterial cultures were cultivated in 1–5 litre flasks at room temperature and under continuous illumination (20 W POWER-GLO aquarium lamps). Agitation was performed by sparging with sterile-filtered air. After 3 weeks the cyanobacterial cells were harvested on Whatman (Maidstone, UK) GF/A filters and the filters were air-dried and stored at -20 °C until extraction. Microcystins were extracted and purified by Dr Lisa Spoof and docent Jussi Meriluoto according to Meriluoto and Spoof (2005b and 2005d).

Toxins produced by *Microcystis* strain PCC 7820 have been reported to be MC-LR, -LY, -LW and -LF (Lawton *et al.*, 1994). Toxins produced by *Microcystis* NIES-107 include 3-demethyl(dm)-MC-RR, MC-RR, MC-YR, 3-dm-MC-LR and MC-LR (Meriluoto *et al.*, 2004). *Anabaena* sp. 90 produces dm-MC-RR, MC-RR, dm-MC-LR and MC-LR (Rapala *et al.*, 1997) and *Anabaena lapponica* 966 produces CYN (Spoof *et al.*, 2006).

#### 3.3 Microcystins

MC-LR and MC-LF were extracted from *Microcystis* PCC 7820 and MC-RR was extracted from *Microcystis* NIES-107. In some experiments MC-LR extracted from a culture of *Anabaena* sp. 90 was used. The microcystins were purified by high-performance liquid chromatography (HPLC) as

described earlier (Meriluoto and Spoof, 2005d). 10 µg of microcystin was dissolved in 1 mL of PBS buffer, and appropriate dilutions were made.

The cyanobacterial extracts studied were *Microcystis* NIES-107 and *Microcystis* PCC 7820. *Microcystis* NIES-107 extract contained 1.3 µg dm-MC-RR, 12.8 µg MC-RR, 2.4 µg MC-YR, and 6.5 µg MC-LR per vial, which was diluted to appropriate concentrations. *Microcystis* PCC 7820 extract contained 10.8 µg MC-LR, 0.94 µg MC-LY, 3.14 µg MC-LW and 2.03 µg MC-LF. CYN was isolated from *Cylindrospermopsis raciborskii* strain CR3 and purified in Prof. G.A. Codd's laboratory (University of Dundee, UK) according to Metcalf and Codd (2005).

### 3.4 Probiotic bacterial strains and cultivation

Eleven commercial bacterial strains were tested for their microcystin removal capacity: *L. rhamnosus* GG (ATCC 53103, Valio Ltd, Helsinki, Finland), *L. rhamnosus* LC-705 (Valio Ltd, Helsinki, Finland), *L. casei* Shirota (obtained from Yakult Singapore Pty. Ltd, Singapore via Prof. Y-K Lee, National University of Singapore, Singapore), *L. fermentum* ME3 (University of Tartu, Estonia), *L. acidophilus* NCFM, *L. plantarum* Lp-115, and *L. salivarius* Ls-33 (Danisco Inc., Madison), *B. lactis* 420 and *B. lactis* HN019 (Danisco Deutschland GmbH, Niebüll, Germany), *B. longum* 46 (DSM 14583) (Laine *et al.*, 2003) and *B. lactis* Bb12 (Chr Hansen Ltd, Hørsholm, Denmark). The bacteria were obtained in commercial lyophilised form. For assays with freshly grown bacteria, the bacteria were cultured in MRS broth (Oxoid, Hampshire, UK) (MRS supplemented with 0.05% w/v cysteine-HCl for bifidobacteria) for 18 h at 37 °C, harvested by centrifugation (3200 × *g*, 4 °C, 20 min) and washed twice with PBS. The viability of the cells was tested on MRS agar plates. Viable counts were determined after incubation for 48 h at 37 °C. Non-viable bacteria were prepared by 95% ethanol treatment of the bacteria for 30 min, by 1 M HCl treatment for 30 min or by heat treatment for 30 min at 90 °C. The bacteria were washed twice with PBS after the treatments. In some experiments the cells were suspended in PBS with or without glucose (1, 2, or 3%). Glucose was added in PBS as a bacterial nutrient. The cell density in all experiments was approximately 10<sup>10</sup> colony forming units (CFU)/mL. Total viable counts of bacterial suspensions were determined prior to incubation by flow cytometry (section 3.4.2).

Two *dadih* lactic acid bacterial strains were tested for their microcystin-removal capacity. The indigenous *dadih* strains were *L. plantarum* strains with the identification numbers IS-10506 and IS-20506 (Gene Bank Accession numbers DQ860148 and DC860149, respectively) from the University of Turku culture collection. The culturing conditions were the same as for the commercial probiotic strains.

#### 3.4.1 Plate counts

Viable counts (CFU/mL) were obtained by plate counting from bacterial suspensions after 0, 24, and 48 h of incubation in PBS containing 100 µg/L MC-LR. The bacterial suspensions were serially diluted in PBS buffer, and appropriate dilutions were spread on MRS agar plates (Oxoid) (MRS

agar supplemented with 0.05% w/v cysteine-HCl for bifidobacteria) and incubated for 48 h at 37 °C (anaerobic conditions for bifidobacteria; 10% H<sub>2</sub>, 10% CO<sub>2</sub>, and 80% N<sub>2</sub>; Concept 400 anaerobic chamber, Ruskinn Technology, Leeds, UK). All plate counts were determined in three independent experiments, and each assay was performed in triplicate.

### 3.4.2 Fluorescent counts

Counts of total, viable, and dead bacteria were obtained by the use of LIVE/DEAD BacLight Bacterial Viability Stain Kit (Molecular Probes, Eugene, OR). The viability kit stain mixture, used for total counts of bacterial population, distinguished live bacterial cells from dead by means of membrane integrity and increased the efficiency of staining to indicate viability (Franks *et al.*, 1998).

Bacterial cells were washed with PBS buffer, and 10 µL was mixed with 1.5 µL of a mixture of SYTO9 and propidium iodide (PI) (1:1), nucleic acid stains from a LIVE/DEAD BacLight. The samples were then vortexed and incubated in darkness for 15 min at room temperature according to the manufacturer's instructions. Immediately prior to analysis, 10 µL of fluorospheres (Molecular Probes) was added to each sample to obtain the absolute bacterial cell count.

Flow cytometric analysis was performed using a BD FACSCalibur flow cytometer (Becton, Dickinson and Co., U.S.) with an air-cooled argon ion laser (488 nm at 15 mW). This standard instrument was equipped with two light scatter detectors that measure forward (FSC) and side scatter (SSC) and two fluorescence detectors (FL1, 525 nm; FL3, 620 nm). Data were stored as list mode files and analysed off-line using the System II V.3 software (Beckman Coulter). Ideally, live bacteria with intact plasma membranes fluoresce green and the dead or injured cells with compromised membranes fluoresce red. In accordance with the manufacturer's instructions, all green cells (SYTO9) were considered viable, and red cells (PI) were considered dead. Viability was determined in two independent experiments, and each assay was performed in duplicate. The percentages of viability were calculated comparing the total viable counts before incubation (t = 0, 100% viability) with the viable cells labelled with SYTO9 (green colour) obtained after 24 and 48 h of incubation at 4 and 37 °C with different concentrations of glucose.

### 3.4.3 Microscopy

Visual inspection of the viability of bacteria during incubation with MC-LR at 37 °C in absence or presence of glucose (0 or 1%) was determined by microscopy. An Olympus epifluorescence microscope BX50 (Olympus, Tokyo, Japan) with filter U-MWIB (excitation, 460–490 nm; emission, 515–700 nm) was used. Images were recorded with a digital camera (model DP-10; Olympus, Hamburg, Germany): green bacteria, viable; red bacteria, non-viable.

### 3.5 *Sphingomonas* strains

*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.

### 3.6 Microcystin removal assay

One millilitre of the freshly grown bacteria suspended in PBS at a concentration of approximately  $10^{10}$  CFU/mL with or without the addition of glucose (1, 2, or 3%) together with 100 µg/L MC-LR were incubated in 1.5 mL borosilicate glass chromatographic vials under continuous reciprocal shaking, 120 rotations per minute (Certomat WR, B. Braun, Melsungen, Germany) at different temperatures (4, 22 or 37 °C) for different times (0, 18, 24, and 48 h). Lyophilised bacteria were used at a concentration of  $8.8 \times 10^9$ – $2.7 \times 10^{10}$  CFU/mL. Microcystin concentrations of 1, 10 and 100 µg/L were used and CYN at a concentration of 100 µg/L. The microcystin concentrations in the experiments with the cyanobacterial extracts were 13 µg/L dm-MC-RR, 128 µg/L MC-RR, 24 µg/L MC-YR, and 65 µg/L MC-LR in *Microcystis* NIES-107 and 108 µg/L MC-LR, 9.4 µg/L MC-LY, 31.4 µg/L MC-LW, and 20.3 µg/L MC-LF in *Microcystis* PCC 7820.

After incubation, 200 µL aliquots were taken and centrifuged ( $12000 \times g$ , 10 min, room temperature) in 300 µL borosilicate glass chromatographic inserts. The supernatants were analysed to determine the residual MC-LR concentration by HPLC or ESI-LC-MS compared with a 100 µg/L microcystin or CYN control in PBS. Each assay was carried out in triplicate. Appropriate microcystin controls without bacteria were also prepared and they showed no reduction of MC-LR during incubation. Plastics were avoided in the preparation of the aqueous microcystin solutions, as adsorption of the toxins on polypropylene surfaces is known to occur (Hyenstrand *et al.*, 2001).

The microcystin removal assay for the *Sphingomonas* strains ACM-3962 and B9 was performed as described above and the potential degradation products were studied and identified by HPLC-DAD and LC-ESI-MS.

### 3.7 Microcystin removal in the presence of bacterial cell extracts

Bacterial cell extracts were prepared for three of the studied probiotic strains: *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), the success of sonication was checked by microscopy, cell debris was removed by centrifugation ( $12000 \times g$ , 10 min, 4 °C) and the supernatant was used for the MC-LR removal assays. MC-LR (final concentration 100 µg/L) was incubated with 1.5 mL of cytosolic cell extract in 1.5 mL borosilicate glass chromatographic vials under continuous reciprocal shaking (120 rotations per minute,



Certomat WR, B. Braun). After 12, 18 and 24 h of incubation at 37 °C aliquots were taken, centrifuged and analysed on HPLC.

### 3.8 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 (3200 × *g*, 20 min), 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 was determined with an enzyme-linked immunosorbent assay (ELISA) QuantiPlate Kit for microcystins according to the manufacturer's instructions (Envirologix, Portland, ME, USA). The amount of toxin found inside the cell pellet was compared with the amount of toxin removed during the 24 h incubation (HPLC results).

### 3.9 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 10<sup>10</sup> CFU/mL together with 100 µg/L MC-LR. 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% glucose addition at 3 h and 6 h (group B); bacterial suspension in PBS and 1% 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 (section 3.14) and 100 µL of sample was used in an enzymatic assay.

### 3.10 Removal of <sup>14</sup>C-labelled MC-LR by probiotic bacteria

Experiments with radiolabelled microcystins were carried out to investigate whether microcystin is transported into the bacterial cells during toxin removal. <sup>14</sup>C-labelled MC-LR was purified from *Microcystis* PCC 7820, which was grown in 1-litre cultures in the presence of sodium <sup>14</sup>C-bicarbonate (50 mCi/mmol, Perkin Elmer Life Sciences, USA) according to Brooks and Codd (1987) and Craig *et al.* (1996). The specific activity of <sup>14</sup>C-MC-LR was 13.4 µCi/mmol. <sup>14</sup>C-MC-LR at a concentration of 1 µg/mL was incubated with the bacterial strain *L. rhamnosus* GG. After 24 h cells were centrifuged and bacterial cell disruption was performed by ultrasonication on ice (Bandelin Sonopuls HD 2070 ultrasonic homogenizer). Both the supernatant and cell extract was analysed for the presence of <sup>14</sup>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 <sup>14</sup>C-MC-LR bound to the cells or present in the supernatant.

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

The activity of cell-associated proteinases of *L. rhamnosus* GG was measured after incubation with different concentrations of protease inhibitors. To check the type of proteinase, 18 h old culture of *L. rhamnosus* GG washed with PBS buffer ( $10^{10}$  CFU/mL) was incubated for 0.5h with different doses of typical inhibitors of metalloprotease (EDTA; 1, 5 and 10 mmol/L), serine protease (PMSF diluted in methanol; 0.5, 5 and 50 mmol/L) and cysteine protease (iodoacetic acid; 0.01, 0.05, 0.1, 0.5 and 1 mmol/L). Additionally, a protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, and sodium EDTA with final concentrations of 2.32, 0.03, 0.2, 0.033 and 10 mmol/L, respectively, was used. To exclude the impact of the inhibitors on the viability of the bacterial cells traditional plate counting using MRS agar plates (section 3.4.1) was performed at the beginning of the experiment and after 6 h of incubation. Based on the results of this experiment, the involvement of EDTA in MC-LR removal was investigated in detail. 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 3.13.

### 3.12 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 Spooft (2005a). The concentration of MC-LR (standard) for preparing the calibration curve ranged between 0.125–4.0  $\mu\text{g/L}$ . Part of the samples, which were analysed by HPLC and in enzymatic assays, were frozen and diluted directly before the experiment to obtain a MC-LR concentration between 0.5–2.0  $\mu\text{g/L}$ . The toxicity values were converted to MC-LR equivalents.

### 3.13 Enzyme assay

The standard peptidase assay was performed at 37 °C with 0.02  $\mu\text{mol/L}$  Suc-Ala-Ala-Phe-AMC in 0.1 M Tris-HCl, pH 7.0 as a substrate and 25  $\mu\text{mol/L}$  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. Appropriate dilutions of the AMC standard (12.5  $\mu\text{mol/L}$ , 6.25  $\mu\text{mol/L}$  and 2.5  $\mu\text{mol/L}$ ) were made fresh daily. 150  $\mu\text{L}$  of substrate was added to 25  $\mu\text{L}$  of sample or standard ( $n=3$ ); 150  $\mu\text{L}$  of Tris buffer was added to 25  $\mu\text{L}$  of cell suspension/extract and 150  $\mu\text{L}$  of substrate with 25  $\mu\text{L}$  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, Finland) at excitation/emission wavelengths of 365/442 nm and the activity was expressed in micromoles of substrate hydrolyzed within 1 h.

### 3.14 HPLC analysis

MC-LR was quantified by HPLC on a Purospher STAR RP-18 endcapped column (55 mm × 4 mm, 3 μm particles; Merck). The mobile phase consisted of a gradient of 0.05% aqueous TFA (solvent A) and 0.05% TFA in acetonitrile (solvent B) with the following linear gradient program: 0 min 25% B, 5 min 70% B, 6 min 70% B, 6.1 min 25% B. The injection interval was 10 min, the injection volume was 30 μL, the flow rate was 1.0 mL/min and the column temperature 40 °C. The HPLC-DAD system consisted of Merck-Hitachi (Darmstadt, Germany) La-Chrom series components: L-7612 degasser, L-7100 quaternary gradient pump, L-7200 autosampler, L-7360 column oven, L-7450A diode-array UV detector operated at 238 nm and D-7000 interface. dm-MC-RR eluted at 3.0 min, MC-RR at 3.2 min, MC-YR at 3.7 min, MC-LR at 3.9 min, MC-LY at 4.9 min, MC-LW at 5.4 min, and MC-LF at 5.6 min.

For experiments with <sup>14</sup>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) was used. The mobile phase consisted of a gradient of 0.05% aqueous TFA (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.

### 3.15 LC-MS analysis

Analysis of samples with lower microcystin concentrations (1–10 μg/L) was performed on LC-ESI-MS. The instrument consisted of an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Waters Micromass (Manchester, UK) Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray interface. MC-LR was quantified on a Purospher STAR RP-18 endcapped column (30 mm × 4 mm, 3 μm particles; Merck). The mobile phase consisted of a gradient of 0.5% aqueous formic acid (solvent A) and acetonitrile (solvent B) with the following linear gradient program: 0 min 25% B, 10 min 70% B, 12 min 90% B, 13 min 90% B, and 13.5 min 25% B. The injection interval was 20 min, the injection volume was 10 μL, the flow rate was 0.5 mL/min and the column temperature was 40 °C. The capillary voltage was set at 3.8 kV and the cone voltage at 40 V (dm-MC-RR and MC-RR) or 75 V (rest of the microcystins). The desolvation gas (nitrogen) temperature and flow rate were set at 300 °C and 650 L/h, respectively. The ion source temperature was set at 150 °C. Ions were detected in the positive electrospray ionisation mode. Selected ion recording (SIR) was used in monitoring the following *m/z*: dm-MC-RR [M+2H]<sup>2+</sup> 512.7, MC-RR [M+2H]<sup>2+</sup> 519.7, MC-LF [M+H]<sup>+</sup> 986.5 and [M+Na]<sup>+</sup> 1008.5, MC-LR [M+H]<sup>+</sup> 995.5, MC-LY [M+H]<sup>+</sup> 1002.5, MC-LW [M+H]<sup>+</sup> 1025.5 and [M+Na]<sup>+</sup> 1047.5, MC-YR [M+H]<sup>+</sup> 1045.5. Data acquisition was performed with MASSLYNX V4.0 software (Micromass).

The mobile phase for CYN consisted of 0.1% formic acid in 1% methanol (solvent A) and acetonitrile (solvent B) with the following linear gradient program: 0 min 0% B, 5 min 0% B, 5.1 min 70% B, 7.0 min 70% B, 7.1 min 0% B. The capillary voltage was set at 3.8 kV and the cone

voltage at 30 V. Ions were detected in the positive electrospray ionisation mode. SIR was used in monitoring  $m/z$  CYN  $[M+H]^+$  416.

In publication IV the instrumentation 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 1200 LC system included a binary pump, vacuum degasser, refrigerated model SL autosampler (set at 10 °C) and column oven. 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 300000 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. 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.

### **3.16 Statistics**

Statistical analysis was performed using the Student's  $t$ -test (Microsoft Office Excel). The probability level of 5% ( $P=0.05$ ) was used to indicate the significance. The  $t$ -test was used to compare the means of the measured variables such as toxin removal percentages. Pearson test was used to indicate the correlation between the proteinase activity and the MC-LR removal.

## 4. RESULTS AND DISCUSSION

The aim of this project was to characterise the potential of probiotic lactic acid bacteria and bifidobacteria in removal of cyanobacterial peptide hepatotoxins and the alkaloid cytotoxin cylindrospermopsin from aqueous solutions. Different physiological conditions possibly affecting the removal efficiency were studied and the mechanism of toxin removal was investigated.

The results described in the results section highlight the main results presented in detail in publications I–IV.

### 4.1 Screening of microcystin removal efficiency of probiotic bacterial strains (I)

Eleven strains of probiotic lactic acid bacteria and bifidobacteria were tested for their MC-LR removal capacities. The results showed a reproducible reduction of MC-LR in solution by the majority of the tested bacterial strains. Strain differences in the removal of microcystins could be noted (Table 4.1). The most efficient removal with lyophilised bacteria was achieved with *B. lactis* Bb12. In addition *L. rhamnosus* strains GG and LC-705, *B. longum* 46 and *B. lactis* 420 showed removal percentages of over 20% within 24 h of incubation. The poorest removal was observed with *L. acidophilus* NCFM (3.5%) and *L. plantarum* Lp-115 (5.7%).

The removal of MC-LR continued during the entire 24 h incubation, which indicates that the removal process is quite slow. Because of the slow removal process, the five strains most efficient in microcystin removal in lyophilised form (*L. rhamnosus* strains GG and LC-705, *B. longum* 46, *B. lactis* 420 and *B. lactis* Bb12) were cultured and assayed to compare the removal efficiencies between lyophilised and freshly grown bacteria (section 4.2). These five strains were also chosen for further studies based on this initial screening experiment.

#### 4.1.1 Effect of pH

The effect of pH in the aqueous solution during incubation of bacterial strains with MC-LR was studied. pH was found to have an influence on toxin removal, with a higher removal percentage observed at neutral pH than at pH 3 (Table 4.1). pH 3 was chosen to examine toxin removal under similar conditions as the low pH in the gastrointestinal tract. pH 3 is an intermediate pH value occurring between the stomach and the upper intestine.

**Table 4.1.** Percentage of MC-LR removed from solution  $\pm$  SD at 37 °C by lyophilised bacteria

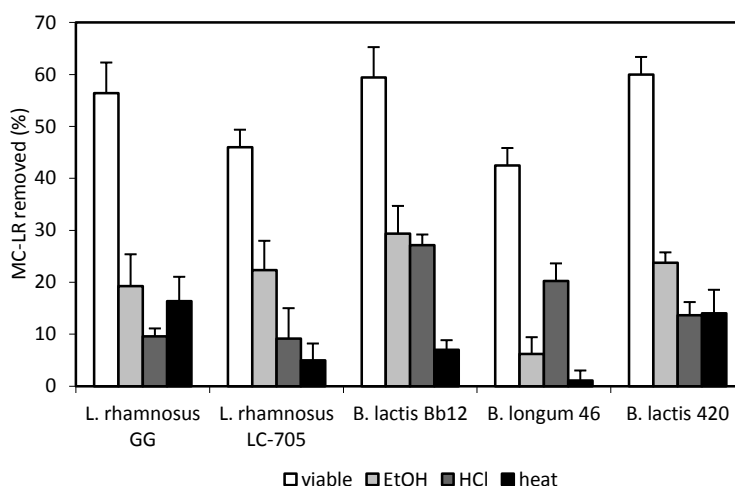
Strain	pH	Incubation time			
		1h	4h	6h	24h
<i>B. lactis</i> 420	3	2.3 $\pm$ 1.3	8.3 $\pm$ 2.0	11.4 $\pm$ 2.3	12.1 $\pm$ 2.0
	7	3.4 $\pm$ 1.9	9.8 $\pm$ 2.6	11.7 $\pm$ 2.9	20.7 $\pm$ 2.5
<i>B. lactis</i> Bb12	3	2.0 $\pm$ 0.5	9.2 $\pm$ 1.4	14.9 $\pm$ 2.2	21.0 $\pm$ 2.7
	7	6.8 $\pm$ 2.0	17.5 $\pm$ 2.6	23.6 $\pm$ 3.9	25.7 $\pm$ 2.4
<i>B. lactis</i> HN019	3	1.1 $\pm$ 1.3	2.3 $\pm$ 1.7	4.9 $\pm$ 2.5	12.0 $\pm$ 4.4
	7	0.3 $\pm$ 1.5	3.3 $\pm$ 0.9	4.3 $\pm$ 1.4	14.6 $\pm$ 2.4
<i>B. longum</i> 46	3	1.3 $\pm$ 0.4	10.7 $\pm$ 2.5	18.1 $\pm$ 1.1	21.2 $\pm$ 2.0
	7	1.7 $\pm$ 0.8	7.7 $\pm$ 0.9	17.4 $\pm$ 2.7	23.0 $\pm$ 3.3
<i>L. acidophilus</i> NCFM	3	0.3 $\pm$ 0.8	0.9 $\pm$ 2.1	1.7 $\pm$ 1.3	3.5 $\pm$ 1.9
	7	0.3 $\pm$ 0.6	3.4 $\pm$ 1.5	4.3 $\pm$ 0.9	9.1 $\pm$ 2.7
<i>L. casei</i> Shirota	3	2.9 $\pm$ 0.8	9.1 $\pm$ 1.6	11.9 $\pm$ 1.5	14.8 $\pm$ 2.7
	7	4.6 $\pm$ 1.2	9.8 $\pm$ 1.4	12.9 $\pm$ 0.7	16.2 $\pm$ 1.9
<i>L. fermentum</i> ME3	3	2.3 $\pm$ 1.3	8.3 $\pm$ 2.0	11.4 $\pm$ 2.3	12.1 $\pm$ 2.0
	7	4.6 $\pm$ 0.5	10.3 $\pm$ 0.7	13.8 $\pm$ 1.6	16.3 $\pm$ 2.1
<i>L. plantarum</i> Lp-115	3	2.3 $\pm$ 1.3	1.4 $\pm$ 2.3	4.2 $\pm$ 1.9	5.7 $\pm$ 2.6
	7	0.3 $\pm$ 0.9	2.1 $\pm$ 2.0	3.3 $\pm$ 1.4	6.0 $\pm$ 2.9
<i>L. rhamnosus</i> GG	3	2.1 $\pm$ 1.1	11.5 $\pm$ 1.2	15.7 $\pm$ 1.6	18.2 $\pm$ 1.8
	7	5.5 $\pm$ 0.7	8.3 $\pm$ 0.9	16.6 $\pm$ 1.5	24.3 $\pm$ 1.6
<i>L. rhamnosus</i> LC-705	3	1.9 $\pm$ 0.7	10.3 $\pm$ 2.0	10.6 $\pm$ 0.9	15.0 $\pm$ 1.1
	7	7.0 $\pm$ 1.1	13.2 $\pm$ 2.7	16.0 $\pm$ 3.6	20.6 $\pm$ 2.8
<i>L. salivarius</i> Ls-33	3	0.5 $\pm$ 1.5	2.5 $\pm$ 2.7	4.4 $\pm$ 1.7	11.5 $\pm$ 2.5
	7	0.2 $\pm$ 0.4	3.7 $\pm$ 2.5	5.3 $\pm$ 1.8	19.4 $\pm$ 2.3

Bacterial concentration  $8.8 \times 10^9 - 2.7 \times 10^{10}$  CFU/mL, initial MC-LR concentration 100  $\mu$ g/L

#### 4.2 Influence of bacterial viability on removal efficiency (I)

To assess the requirement of viability for removal of MC-LR, the bacteria were inactivated with different treatments (ethanol, acid or heat treatment) and the removal capacity of the non-viable bacteria was then assessed and compared with that of viable bacteria (Fig. 4.1). For all five strains studied (*L. rhamnosus* strains GG and LC-705, *B. longum* 46, *B. lactis* 420 and *B. lactis* Bb12), viable bacteria were shown to be more effective in microcystin removal than non-viable bacteria. The removal percentages observed with viable bacteria were 45–65%, while non-viable bacteria showed toxin-removal percentages of below 10–20%. Freshly cultured bacteria were shown to be more effective in microcystin removal than lyophilised bacteria (Table 4.1). Non-viable bacteria could still remove MC-LR to some extent. This could be explained by the fact that the treatments can either alter the surface properties of the bacteria, or the microcystins could be bound to exposed internal surfaces more suitable for toxin removal, as the bacterial cells probably do not stay intact and cell lysis is possible due to the treatment procedures.

Lactic acid bacteria have previously been reported to bind various contaminants and toxins, such as heavy metals and aflatoxins. It appears that AFB<sub>1</sub> is bound to the surface components of lactic acid bacteria (Haskard *et al.*, 2001). Heat- and acid-treated bacteria have also been shown to be effective in AFB<sub>1</sub>-removal. The effectiveness of non-viable bacteria suggests that AFB<sub>1</sub> is not removed by bacterial metabolism, but rather is physically bound probably to the bacterial cell wall or cell wall components (El-Nezami *et al.*, 1998b; Haskard *et al.*, 2000; Lee *et al.*, 2003). The mechanism of removal of heavy metals has also been shown to be physical adsorption to the cell surface (Halttunen *et al.*, 2007a). Therefore it can be concluded that the cyanotoxin-removal process by probiotic bacteria differs from the previously reported removal processes of heavy metals and aflatoxin.



**Figure 4.1.** Influence of viability on removal of MC-LR from solution. Non-viable bacteria were prepared by ethanol (95%, 30 min), acid (1 M HCl, 30 min) or heat (90 °C, 30 min) treatment. Initial MC-LR concentration 100 µg/L, bacterial concentration before treatment 10<sup>10</sup> CFU/mL, incubation time 24 h, temperature 37 °C, pH 7, average ± SD, n = 3.

#### 4.3 Effect of incubation temperature on removal efficiency (I)

The removal of MC-LR at different temperatures was also assessed. As can be seen in Table 4.2, the removal of MC-LR was shown to be temperature dependent, with the highest removal observed at 37 °C for all studied strains. This was significantly different ( $P < 0.05$ ) from the removal percentages obtained at 22 °C and 4 °C. Optimal removal was achieved at 37 °C with all five strains. At 4 °C, practically no removal of MC-LR could be observed. The removal percentages increased with increasing temperature. This can be explained by the fact that at 4 °C, the bacterial cells are metabolically inactive, but at 22 and 37 °C, the mesophilic bacteria become metabolically active, which is required for enzymatic activity and metabolism.

**Table 4.2.** Percentage of MC-LR removed from solution  $\pm$  SD at 4 °C, 22 °C and 37 °C by five strains of probiotic bacteria

Strain	Incubation temperature		
	4 °C	22 °C	37 °C
<i>L. rhamnosus</i> GG	2.6 $\pm$ 5.6	17.7 $\pm$ 4.5	49.3 $\pm$ 2.6
<i>L. rhamnosus</i> LC-705	2.0 $\pm$ 3.0	16.2 $\pm$ 3.3	50.3 $\pm$ 5.1
<i>B. longum</i> 46	2.9 $\pm$ 4.4	18.1 $\pm$ 2.9	47.0 $\pm$ 3.2
<i>B. lactis</i> 420	5.5 $\pm$ 2.9	17.3 $\pm$ 2.6	47.7 $\pm$ 2.0
<i>B. lactis</i> Bb12	4.1 $\pm$ 4.2	22.9 $\pm$ 2.2	58.1 $\pm$ 4.3

Bacterial concentration  $10^{10}$  CFU/mL, initial MC-LR concentration 100  $\mu$ g/L, 24 h incubation, pH 7

#### 4.4 Removal of different microcystins and cylindrospermopsin (II)

The results of this study show that specific strains of probiotic bacteria were effective in the elimination of several different microcystins and cylindrospermopsin from solution. Table 4.3 shows the removal of MC-LR, -RR and -LF (one microcystin variant per experiment) by the probiotic bacterial strains *L. rhamnosus* GG and LC-705, *B. lactis* 420 and Bb12 and *B. longum* 46 after 24 h of incubation at 37 °C. All strains showed removal percentages above 45% for all three microcystins after 24 h when incubated with one microcystin variant per experiment. The removal of cylindrospermopsin was shown to be somewhat less efficient, around 20–30% for all tested strains (Table 4.4).

Simultaneous removal of several toxins present in cyanobacterial extracts was also investigated. The time course for the removal of microcystins present in the cyanobacterial extracts *Microcystis* NIES-107 and *Microcystis* PCC 7820 by the probiotic strain *L. rhamnosus* GG is shown in Fig. 4.2a and b, respectively. The removal of all microcystins increased over time. The rate of removal was shown to be at highest during the initial hours of incubation and then gradually decreased. The removal of the microcystins present in *Microcystis* NIES-107 after 24 h of incubation was around 45–60% of total microcystin and for microcystins present in *Microcystis* PCC 7820 around 50–75%. The toxin-removal was thus shown to be efficient also when several different microcystins were present in the solution. This indicates that there is no competition taking place among the toxins during incubation with probiotic bacteria.

The most efficient removal of the cyanotoxins studied was observed for MC-LW and MC-LF (more than 70% within 24 h). These microcystins have a more hydrophobic structure compared to MC-RR or MC-LR. MC-LF and MC-LW are both analogues of MC-LR with phenylalanine and tryptophan in place of arginine, and they have been suggested to be more cell-permeable than the more hydrophilic microcystins (Kuiper-Goodman *et al.*, 1999). MC-LF and MC-LW have also been shown to have higher surface activities in lipid model membrane monolayers as compared to that of the more hydrophilic MC-LR (Vestervik and Meriluoto, 2003) and thus they might more easily



penetrate the membrane. These microcystins could, therefore, be more easily available for removal by the bacteria.

The probiotic bacteria were shown to be relatively effective in cylindrospermopsin removal, but the removal efficacy was lower than that observed for microcystins. The structure of the cylindrospermopsin molecule, a tricyclic guanidine moiety attached to a hydroxymethyluracil, could affect the possible uptake and degradation of the toxin. Microcystins consist of a peptide ring, which differs from the cylindrospermopsin structure.

**Table 4.3.** Percentage of MC-LR, MC-RR or MC-LF separately removed from solution  $\pm$  SD at 37 °C after 24 h incubation

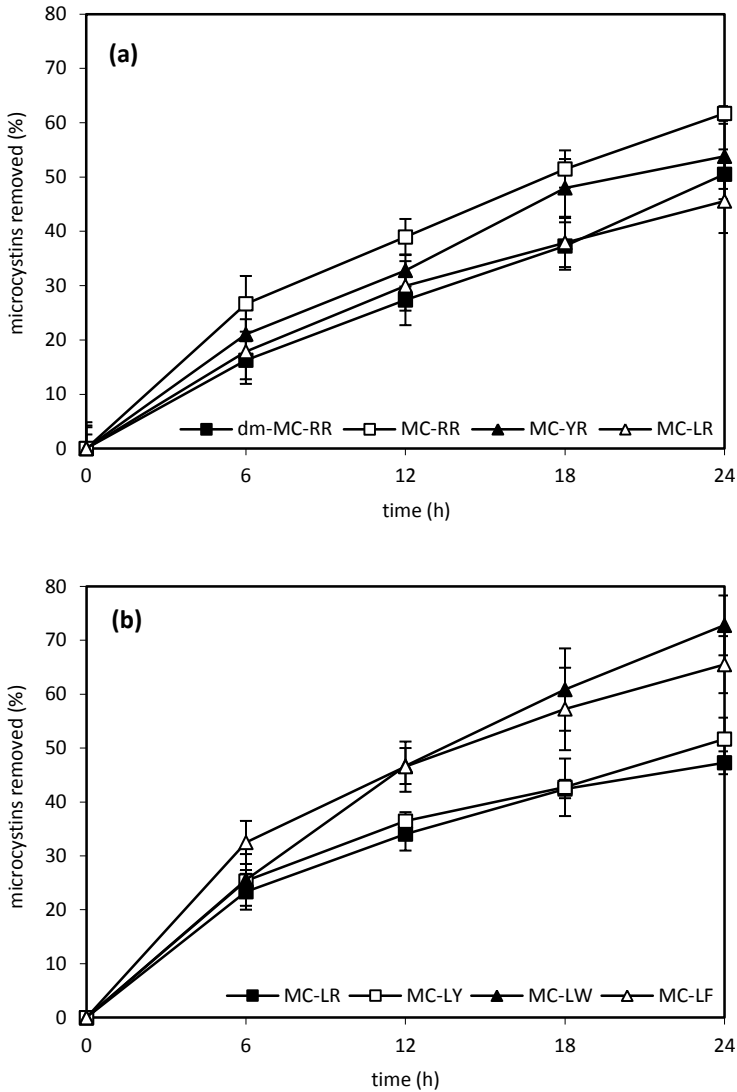
Strain	Microcystin		
	MC-LR	MC-RR	MC-LF
<i>L. rhamnosus</i> GG	60.3 $\pm$ 3.5	56.0 $\pm$ 4.1	72.6 $\pm$ 7.1
<i>L. rhamnosus</i> LC-705	50.3 $\pm$ 2.4	62.8 $\pm$ 5.4	77.4 $\pm$ 1.6
<i>B. longum</i> 46	47.0 $\pm$ 4.2	45.8 $\pm$ 5.9	56.0 $\pm$ 4.8
<i>B. lactis</i> 420	49.7 $\pm$ 3.7	59.7 $\pm$ 3.4	47.8 $\pm$ 2.1
<i>B. lactis</i> Bb12	51.3 $\pm$ 3.2	51.4 $\pm$ 4.6	51.8 $\pm$ 5.4

Bacterial concentration  $10^{10}$  CFU/mL, microcystin concentration 100  $\mu$ g/L

**Table 4.4.** Percentage of CYN removed from solution  $\pm$  SD at 37 °C

Strain	Incubation time			
	5 min	12 h	18 h	24 h
<i>L. rhamnosus</i> GG	1.3 $\pm$ 2.0	20.3 $\pm$ 5.2	25.6 $\pm$ 3.5	30.6 $\pm$ 4.1
<i>L. rhamnosus</i> LC-705	1.9 $\pm$ 1.2	15.1 $\pm$ 2.8	19.0 $\pm$ 4.6	25.4 $\pm$ 2.8
<i>B. longum</i> 46	0.7 $\pm$ 1.4	20.4 $\pm$ 3.6	27.7 $\pm$ 2.9	31.6 $\pm$ 2.9
<i>B. lactis</i> 420	0.5 $\pm$ 0.6	16.1 $\pm$ 4.0	20.1 $\pm$ 4.4	23.0 $\pm$ 3.1
<i>B. lactis</i> Bb12	0.5 $\pm$ 0.9	15.6 $\pm$ 3.8	16.2 $\pm$ 3.5	22.6 $\pm$ 3.4

Bacterial concentration  $10^{10}$  CFU/mL, CYN concentration 100  $\mu$ g/L

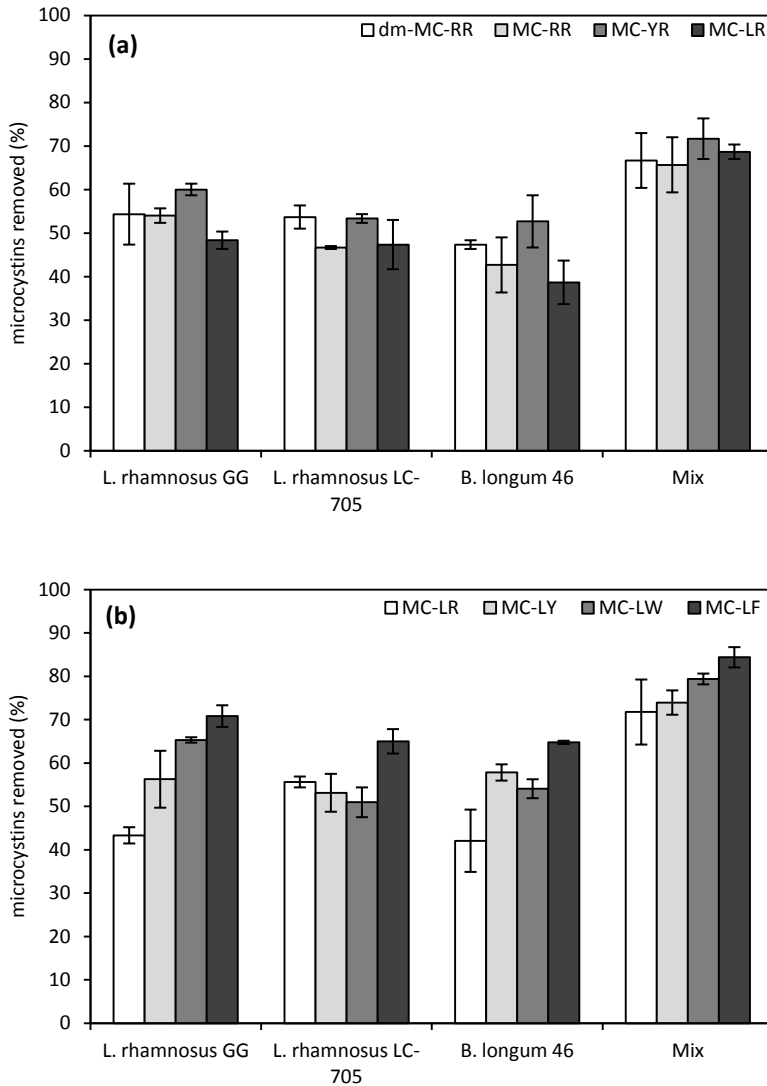


**Figure 4.2.** Time course for removal of microcystins in (a) *Microcystis* NIES-107 and (b) *Microcystis* PCC 7820 by *L. rhamnosus* GG. Initial concentration of microcystins in *Microcystis* NIES-107: 13  $\mu\text{g/L}$  dm-MC-RR, 128  $\mu\text{g/L}$  MC-RR, 24  $\mu\text{g/L}$  MC-YR, and 65  $\mu\text{g/L}$  MC-LR, and in *Microcystis* PCC 7820: 108  $\mu\text{g/L}$  MC-LR, 9.4  $\mu\text{g/L}$  MC-LY, 31.4  $\mu\text{g/L}$  MC-LW, and 20.3  $\mu\text{g/L}$  MC-LF, bacterial concentration  $10^{10}$  CFU/mL, temperature 37 °C, average  $\pm$  SD, n = 3.

#### 4.5 Comparison of microcystin removal by probiotic strains separately and in combination (II)

Fig. 4.3 shows the comparison of microcystin removal of three probiotic strains separately and in combination. The question of this study was whether a combination of several probiotic strains could enhance their microcystin-removal efficiency. The probiotic strains *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46 were incubated with the cyanobacterial extracts *Microcystis* NIES-107 and PCC 7820 at a bacterial concentration of  $10^{10}$  CFU/mL separately or in combination, where the total bacterial cell density was  $10^{10}$  CFU/mL (labelled mix in Fig. 4.3). The removal percentages of the individual strains after 24 h of incubation were calculated and compared to the mixture of the three strains. With the probiotic mixture, microcystin-removal percentages of up to 80% could be observed. The results show that the removal efficiency was improved with a mixture of the strains and the removal percentages observed for the microcystins were significantly higher as compared to the individual strains ( $P < 0.05$ ).

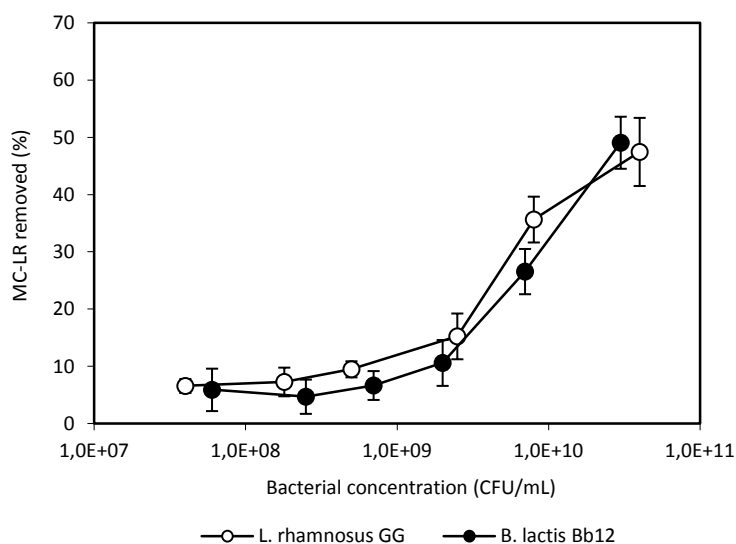
As a result of variations in toxin-removal efficiency of different probiotic strains, combinations of bacteria may be beneficial for efficient removal of microcystins from solution. The functionality of a multistrain probiotic could be more effective and more consistent than that of a single strain (Timmerman *et al.*, 2004). A mixture of several strains could therefore enhance their efficiency and improve their toxin removal abilities.



**Figure 4.3.** Removal of MC-LR by individual probiotic strains and a mixture of three strains. (a) Removal of microcystins in *Microcystis* NIES-107 by *L. rhamnosus* GG, *L. rhamnosus* LC-705, *B. longum* 46 and mix, (b) removal of microcystins in *Microcystis* PCC 7820 by *L. rhamnosus* GG, *L. rhamnosus* LC-705, *B. longum* 46 and mix. Toxin concentrations same as in Fig. 4.2, bacterial concentration  $10^{10}$  CFU/mL, 24 h incubation, temperature 37 °C, average  $\pm$  SD, n = 3.

#### 4.6 Effect of bacterial cell density on toxin-removal efficiency (I)

To investigate the role of the probiotic bacterial cell density, a range of bacterial cell densities were screened (approximately  $5 \times 10^7$ – $5 \times 10^{10}$  CFU/mL) and tested for their microcystin-removal efficiencies. The removal of MC-LR was shown to be dependent on the bacterial cell density, with a minimum of  $3 \times 10^9$  CFU/mL of strain *L. rhamnosus* GG required for significant MC-LR removal ( $P < 0.05$ ) (Fig. 4.4; results shown for two probiotic strains; *L. rhamnosus* GG and *B. lactis* Bb12). The removal of MC-LR was enhanced with increasing bacterial cell density.



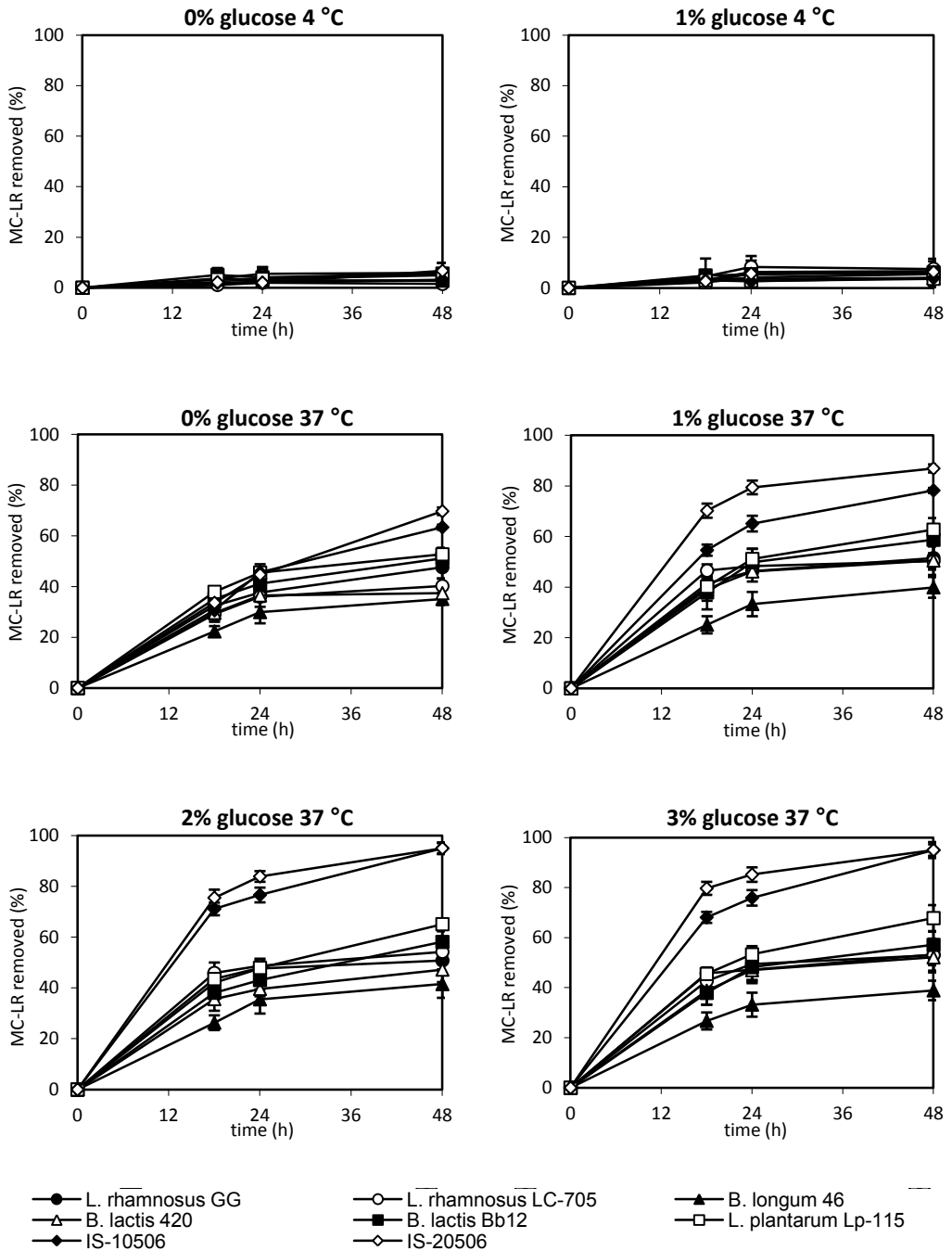
**Figure 4.4.** Effect of bacterial concentration on removal of MC-LR. Initial MC-LR concentration 100  $\mu$ g/L, incubation time 24 h, temperature 37°C, pH 7, average  $\pm$  SD, n = 3.

#### 4.7 Effect of glucose on microcystin removal (III)

The aim of this study was to investigate the main factors influencing the metabolic activity and enhancing the viability of probiotic strains in removal of MC-LR. In this study, in addition to the five commercial probiotic strains used in previous experiments, also the *L. plantarum* strain Lp-115 as well as two strains isolated from a traditional Indonesian fermented milk product dadih (*L. plantarum* IS-10506 and IS-20506) were investigated. 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 and capped with banana leaves (Akuzawa and Surono, 2002). Different bacteria, mainly lactobacilli (*L. plantarum*, *L. casei*, and others), enterococci (*E. faecium*), and some pediococci can be found in the fermented dadih product (Surono and Hosono, 1996; Surono, 2003).

The specific aim of this study was to assess the role of glucose in activating the metabolism of the probiotic bacteria. Fig. 4.5 shows the removal percentages of MC-LR for all tested strains at 4 °C and 37 °C when suspended in PBS alone, which cannot provide any nutrients to the bacteria, and in PBS complemented with 1–3% glucose. The temperatures were chosen to test the influence of bacterial metabolic activity on the removal efficiency. A temperature of 4 °C was chosen as the temperature at which the probiotic bacteria are metabolically inactive. A temperature of 37 °C is the optimal growth temperature for the commercial probiotic bacteria, while the optimal growth temperature for the *L. plantarum* strains is 30 °C (De Angelis *et al.*, 2004). In addition, the metabolic activity of the bacteria was influenced by the glucose addition.

Practically no removal of MC-LR could be observed at 4 °C, whereas at 37 °C all bacterial strains tested were able to remove MC-LR (Fig. 4.5) although the removal efficiency was dependent on the strain and on glucose addition. The results suggest that bacterial metabolic activity plays an important role in toxin removal because at 37 °C, where the bacteria are able to stay metabolically active, the removal percentages were highest ( $P < 0.05$ ) for all tested strains. The highest MC-LR removal percentages were obtained at 37 °C using the *L. plantarum* strains isolated from dadih fermented milk. Furthermore, the glucose addition improved the removal efficiencies of all tested strains at 37 °C by enhancing both the removal rate and the amount of MC-LR removed after 48 h of incubation. In general, glucose had a positive effect on the removal abilities, with higher removal percentages observed with the addition of 1% glucose. The addition of 2 or 3% glucose did not improve or affect the removal further. At 4 °C, glucose did not have any effect on the removal efficiency.



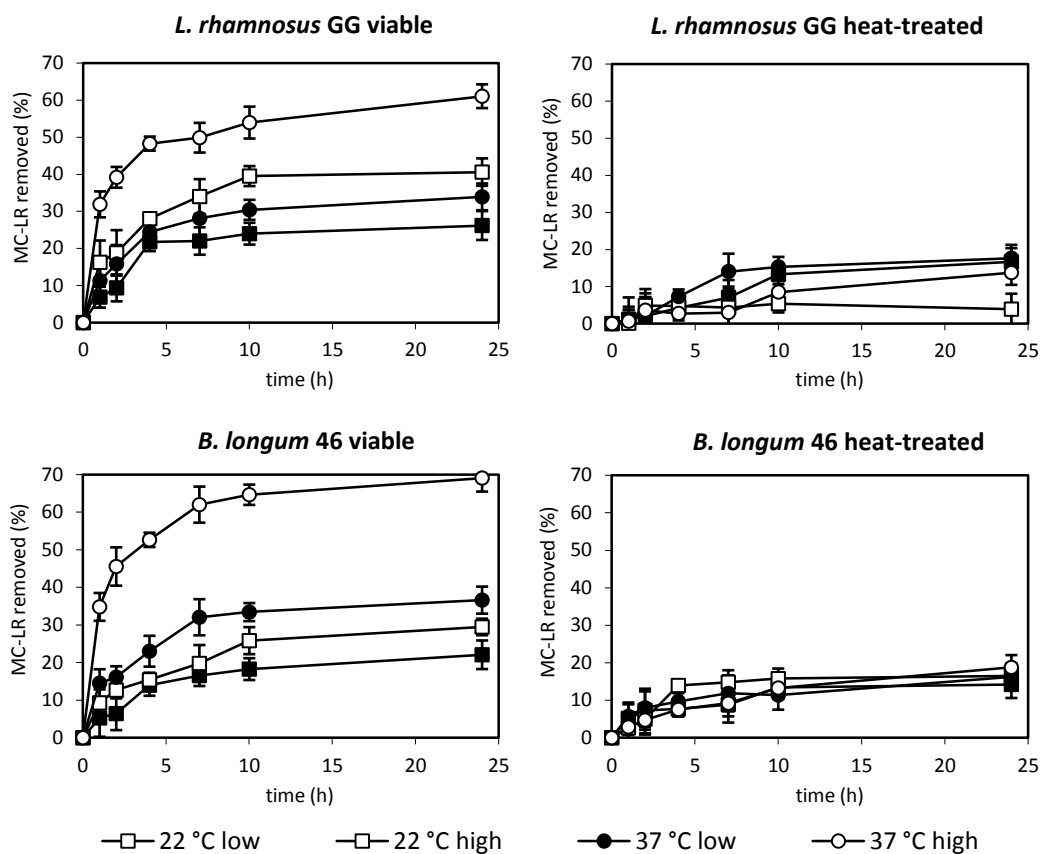
**Figure 4.5.** Percentage of MC-LR removed by freshly cultured bacteria incubated in PBS or PBS containing glucose. Initial MC-LR concentration, 100  $\mu\text{g/L}$ ; incubation times, 0, 18, 24, and 48 h; temperatures 4 °C and 37 °C; 0–3% glucose; average  $\pm$  SD,  $n = 3$ .

#### 4.8 Time course for MC-LR removal (I)

The time course for MC-LR removal (Fig. 4.6) by viable and non-viable bacteria (*L. rhamnosus* GG and *B. longum* 46) at 22 and 37 °C showed that the removal was the highest during the initial 4 h and then decreased gradually. Viable bacteria were efficient in MC-LR removal, while the removal percentages for heat-treated bacteria remained below 20% for both tested strains. With higher bacterial concentrations, higher removal percentages were observed. The removal percentages obtained with non-viable bacteria were low even after an incubation of 24 h.

The rate of microcystin removal from solution was slow compared with that of the previously reported aflatoxin or heavy metal removal, which suggests that the removal mechanisms are not the same. Heavy metals are removed within minutes of incubation (Halttunen *et al.*, 2007a) and the AFB<sub>1</sub>-removal has also been reported to be a rapid process (El-Nezami *et al.*, 1998a; Peltonen *et al.*, 2001). The rapid removal process as well as the efficiency of non-viable bacteria suggested that the removal mechanism of heavy metals and AFB<sub>1</sub> is physical binding of the contaminants to the probiotic bacterial cells. The exact mechanism of cyanotoxin removal after this study could not be reported, but the inefficiency of inactivated bacteria as well as the significance of optimal incubation temperature suggested a metabolic activity, such as toxin degradation by the bacteria.



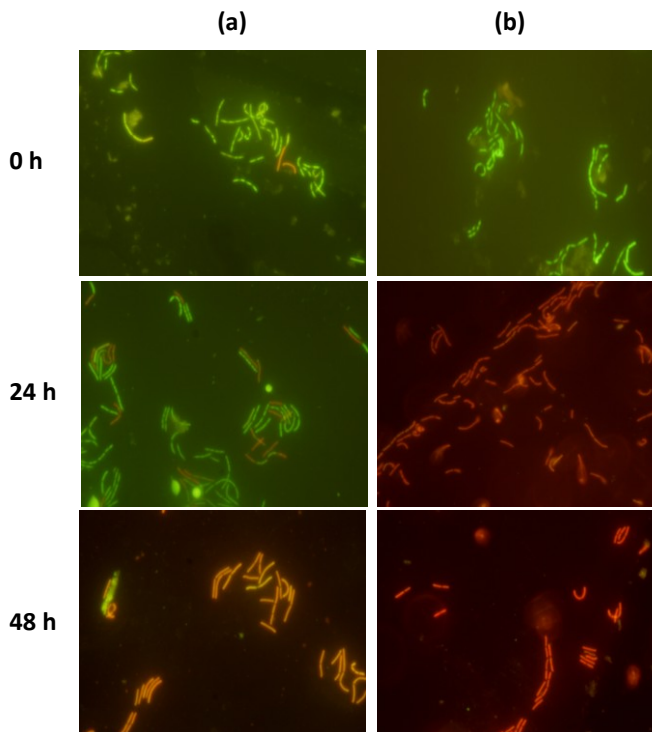


**Figure 4.6.** Time course for removal of MC-LR by viable and non-viable bacteria. Non-viable bacteria were prepared by heat treatment (90 °C, 30 min). Initial MC-LR concentration 100 µg/L, temperatures 22 and 37 °C, pH 7, average ± SD, n = 3. Bacterial concentrations  $5 \times 10^9$  CFU/mL (low) and  $5 \times 10^{10}$  CFU/mL (high).

#### 4.9 Flow cytometric analysis of bacterial cell viability (III)

One specific aim was to assess the importance of cell viability in MC-LR removal. The viability of all bacterial strains tested was analysed by both plate counting and the LIVE/DEAD staining kit using flow cytometry. Ideally, live bacteria with intact plasma membranes fluoresce green and the dead or injured cells with compromised membranes fluoresce red. In accordance with the manufacturer's instructions, all green cells (SYTO9) were considered viable, and red cells (PI) were considered dead. The total bacterial population was enumerated by counting green (SYTO9) and red microorganisms (PI) using flow cytometry and the results were confirmed by an epifluorescence microscope (Fig. 4.7; results shown only for *L. rhamnosus* GG). The microscopic

images showed that in the absence of glucose most of the cells stayed viable until 24 h of incubation, while the presence of glucose reduced cell viability markedly already after 24 h of incubation. The images also support the conclusion that the bacteria become stressed as a result of higher metabolic activity when glucose is added. During incubation, the bacterial strains consume glucose and as a consequence produce lactic acid, which in turn causes cell stress as a result of the decrease in pH. Consequently, the cells die faster in the presence of glucose. At 37 °C, the metabolic activity rate is higher in the presence of glucose and the viable cells may experience stress as shown by the decrease of viability.

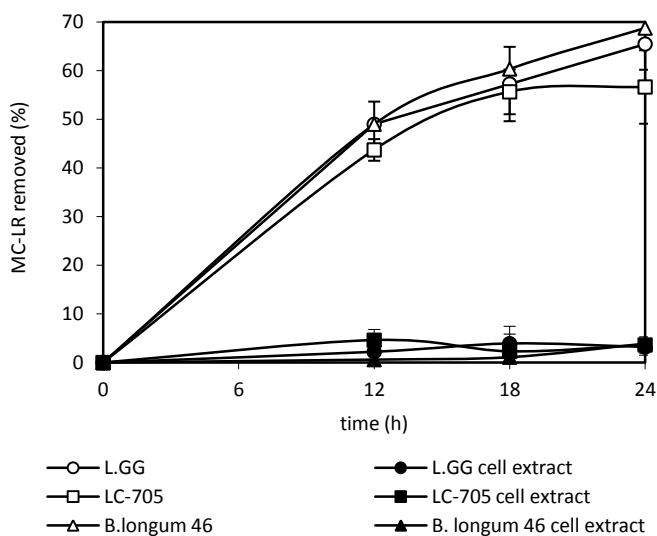


**Figure 4.7.** Viability of bacteria (*L. rhamnosus* GG) after 0, 24, and 48 h of incubation at 37 °C in the absence or presence of 1% glucose determined by microscopy. Green bacteria: viable; red bacteria: non-viable. (a) 0% glucose and (b) 1% glucose.

#### 4.10 Removal of MC-LR by whole cells versus cell extracts (IV)

The aim of this experiment was to study whether microcystins can be removed by extracts of the probiotic bacteria or if viable intact bacteria are needed. The cell extracts were prepared by culturing the bacteria, disrupting the cell structure by ultrasonication (checked by microscopy) followed by centrifugation ( $12000 \times g$ , 10 min) and use of supernatant for incubation with MC-LR.

Fig. 4.8 shows the comparison of MC-LR removal by cell solutions of *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46 and the cytosolic cell extracts of the same strains. Incubation with bacterial cell solutions ( $10^{10}$  CFU/mL) showed a 55–70% reduction of MC-LR within 24 h at 37 °C, which was in accordance with the previous studies. 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 the bacterial cells is needed for efficient microcystin removal.



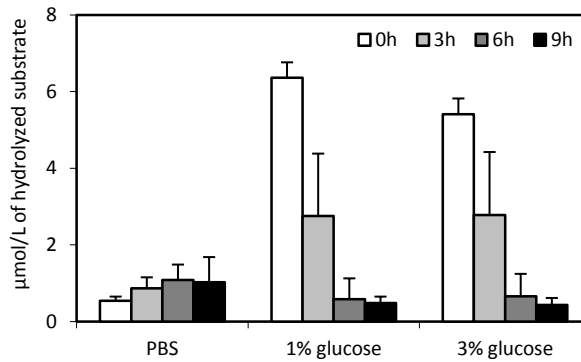
**Figure 4.8.** Removal of MC-LR by probiotic bacterial cells versus cytosolic cell extracts (*L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46). Initial MC-LR concentration 100 µg/L, bacterial concentration  $10^{10}$  CFU/mL, temperature 37 °C; average  $\pm$  SD, n = 3.

#### 4.11 Activity of cell-envelope proteinases and MC-LR removal (IV)

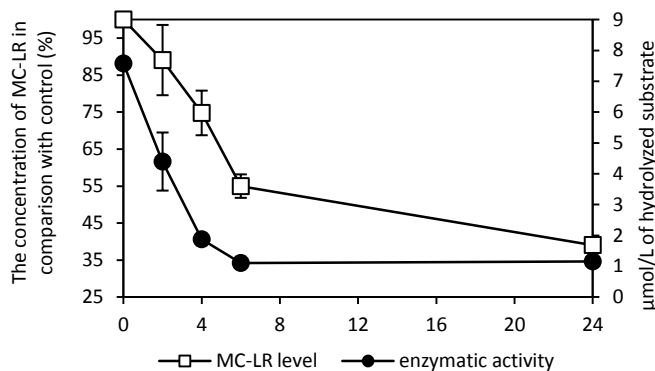
The participation of cell-envelope proteinases in microcystin removal was investigated. Following standard peptidase assay no proteolytic activity against Suc–Ala–Ala–Phe–AMC was found in the supernatant obtained by centrifugation of the bacterial cell cultures of the investigated strains *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46. Enzymatic activity was found only in the cell suspensions. Enzymatic activity was much higher when measured in cell suspensions incubated with glucose than after incubation with PBS (Fig. 4.9) and the activity decreased during incubation. After 24 h the activity had disappeared almost completely (not shown).

Simultaneous analysis of the enzymatic activity and the rate of MC-LR removal indicated that these parameters are closely related and both decreased with time (Fig. 4.10; results shown for *L.*

*rhamnosus* GG; analogous results for *L. rhamnosus* LC-705 and *B. longum* 46 in publication IV). The enzymatic activity and the concentration of MC-LR were lowered within the first six hours of incubation. After 6 h of incubation both the rate of toxin removal and the rate of substrate hydrolysis were slowed down. Significant correlation between enzymatic activity and the removal of MC-LR was found for three investigated strains ( $r = 0.88$ ,  $F = 27.18$ ,  $P < 0.01$  for *L. rhamnosus* GG;  $r = 0.97$ ,  $F = 126.5$ ,  $P < 0.01$  for *L. rhamnosus* LC-705; and  $r = 0.95$ ,  $F = 29.5$ ,  $P < 0.01$  for *B. longum* 46).



**Figure 4.9.** 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. Cell suspension used in enzyme assays was  $10^{10}$  CFU/mL; average  $\pm$  SD,  $n = 3$ .



**Figure 4.10.** The correlation of changes in MC-LR removal and proteinase activity measured within 24 h of incubation for *L. rhamnosus* GG. Initial MC-LR concentration 100 µg/L, bacterial concentration  $10^{10}$  CFU/mL, temperature 37 °C, average  $\pm$  SD,  $n = 3$ .

#### 4.12 Studies with protease inhibitors (IV)

The activity of cell-associated proteinases of *L. rhamnosus* GG was measured after incubation with different concentrations of protease inhibitors. For EDTA the concentration of inhibitor that caused considerable reduction of measured activity was 10 mmol/L (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 with 10 mmol/L concentration of EDTA (Table 4.5 and Fig. 4.11a). It suggested that the main proteolytic activity observed for this strain was due to metallo-enzymes. PMSF did not change the activity of cell-envelope proteinases even at a high concentration (50 mmol/L), whereas almost complete abolition of proteolytic activity was observed after incubation with iodoacetic acid (Table 4.5).

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 had been removed in the presence of EDTA and protease inhibitor cocktail, respectively (Fig. 4.11b). A correlation between proteinase activity and MC-LR removal was also found when these parameters were measured simultaneously (Fig. 4.12).

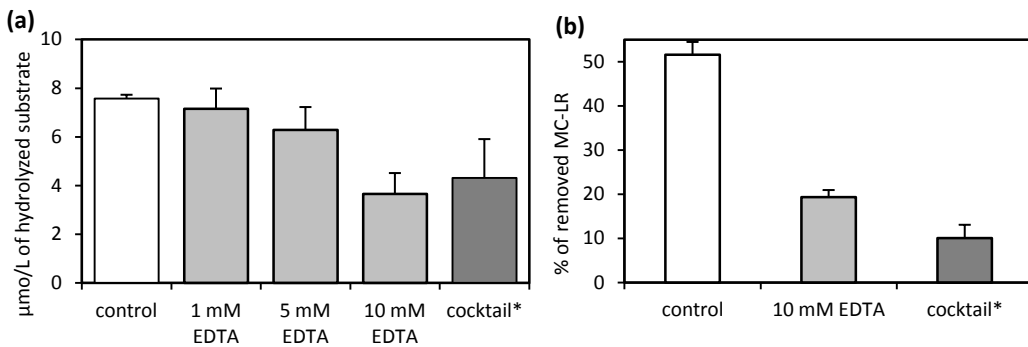
Experiments with different proteinase inhibitors allowed concluding that metallo-peptidases are the main enzymes anchored to the cell wall. This finding is in agreement with other studies, because extracellular proteinases identified in lactic acid bacteria are generally metalloenzymes or serine proteases (Stefanitsi *et al.*, 1995; Guo *et al.*, 2009). EDTA was shown to inhibit MC-LR removal. All these results are in agreement with previous findings that EDTA affects the proteinase activity of probiotic bacteria and that this activity is strongly correlated with the process of MC-LR removal.

**Table 4.5.** 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 or protease inhibitor cocktail

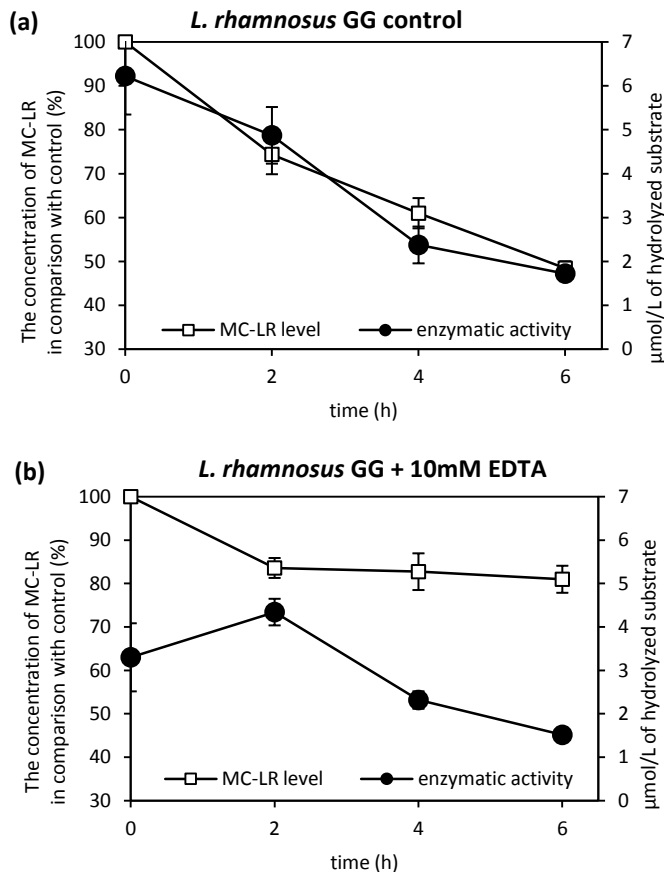
Type of inhibitor	Control (PBS + 1% glucose)		Cells incubated with inhibitor	
	proteolytic activity (μmol/L of hydrolyzed substrate/h)	cell counts (CFU/mL)	proteolytic activity (μmol/L of hydrolyzed substrate/h)	cell counts (CFU/mL)
EDTA (10 mmol/L)	7.57 ± 0.88	3.0 × 10 <sup>10</sup>	3.66 ± 0.78	2.1 × 10 <sup>10</sup>
PMSF (50 mmol/L)	7.57 ± 0.88	2.2 × 10 <sup>10</sup>	7.24 ± 1.22	-
iodoacetic acid (0.1 mmol/L)	7.57 ± 0.88	2.8 × 10 <sup>10</sup>	1.14 ± 0.43	below 10 <sup>5</sup>
protease inhibitor cocktail <sup>a</sup>	6.87 ± 0.39	3.2 × 10 <sup>10</sup>	4.09 ± 0.14	2.0 × 10 <sup>10</sup>

Only the values for concentrations causing significant reduction of proteolytic activity are shown; for PMSF the value for highest concentration used is presented.

<sup>a</sup> final concentrations of inhibitors were 2.32 mmol/L AEBSF, 0.03 mmol/L pepstatin A, 0.2 mmol/L E-64, 0.033 mmol/L bestatin and 10 mmol/L sodium EDTA



**Figure 4.11.** 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 EDTA; average  $\pm$  SD,  $n = 3$ . \*values of measured parameters in the presence of a protease inhibitor cocktail containing 10 mmol/L of EDTA.

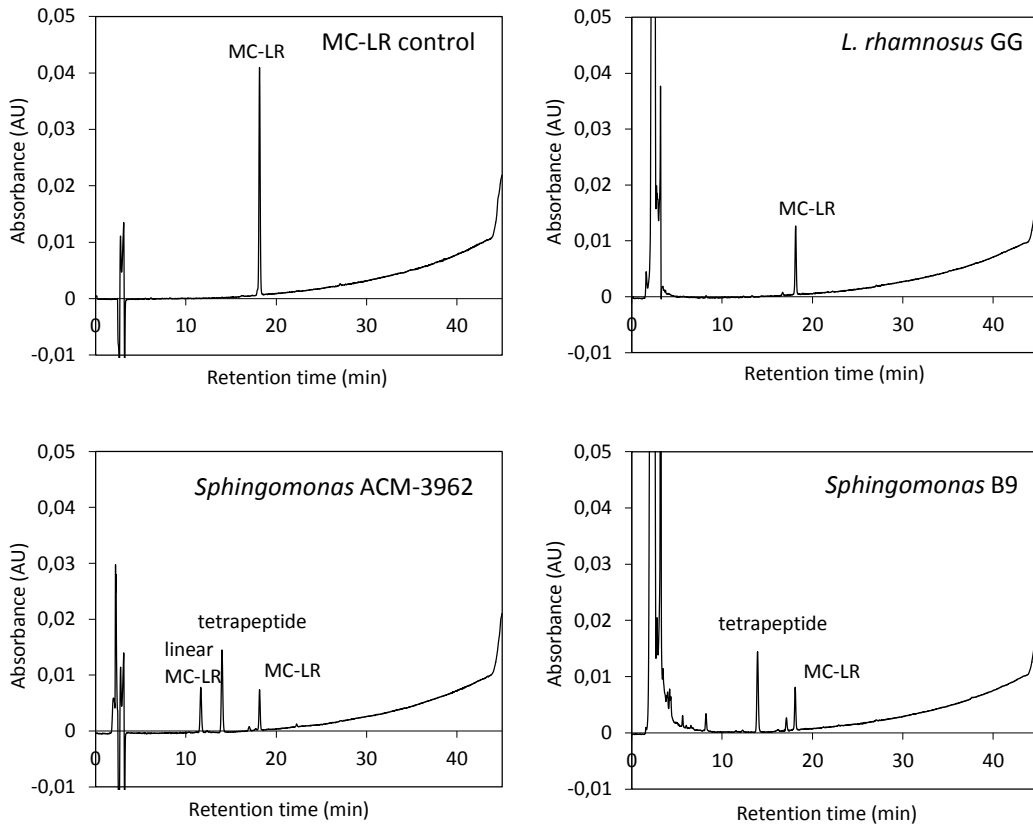


**Figure 4.12.** MC-LR level and proteinase activity of *L. rhamnosus* GG incubated in (a) standard condition and (b) with 10 mmol/L EDTA; average  $\pm$  SD,  $n = 3$ .

#### 4.13 Comparison of MC-LR degradation products for *Sphingomonas* strains and probiotic bacteria (IV)

Different bacterial strains, other than lactobacilli or bifidobacteria, have previously been shown to be able to degrade microcystins enzymatically. Bacterial degradation of microcystins has been reported for strains of *Sphingomonas* (Bourne *et al.*, 1996 and 2001; Harada *et al.*, 2004; Ishii *et al.*, 2004). In this study, 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 were identified. Figure 4.13 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, whereas with the probiotic strain 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. 4.13).

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 the tetrapeptide (Adda-Glu-Mdha-Ala-OH). The linear MC-LR and the tetrapeptide were seen in the HPLC chromatogram of the ACM-3962 strain, but for B9 the linearized MC-LR had already been further degraded and only the tetrapeptide was seen (Fig. 4.13). The products and degradation patterns (not shown) were identified by HCT Ultra Ion Trap LC-MS (Fig. 4.14 and Table 4.6). 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 identified from samples incubated with the probiotic strain.



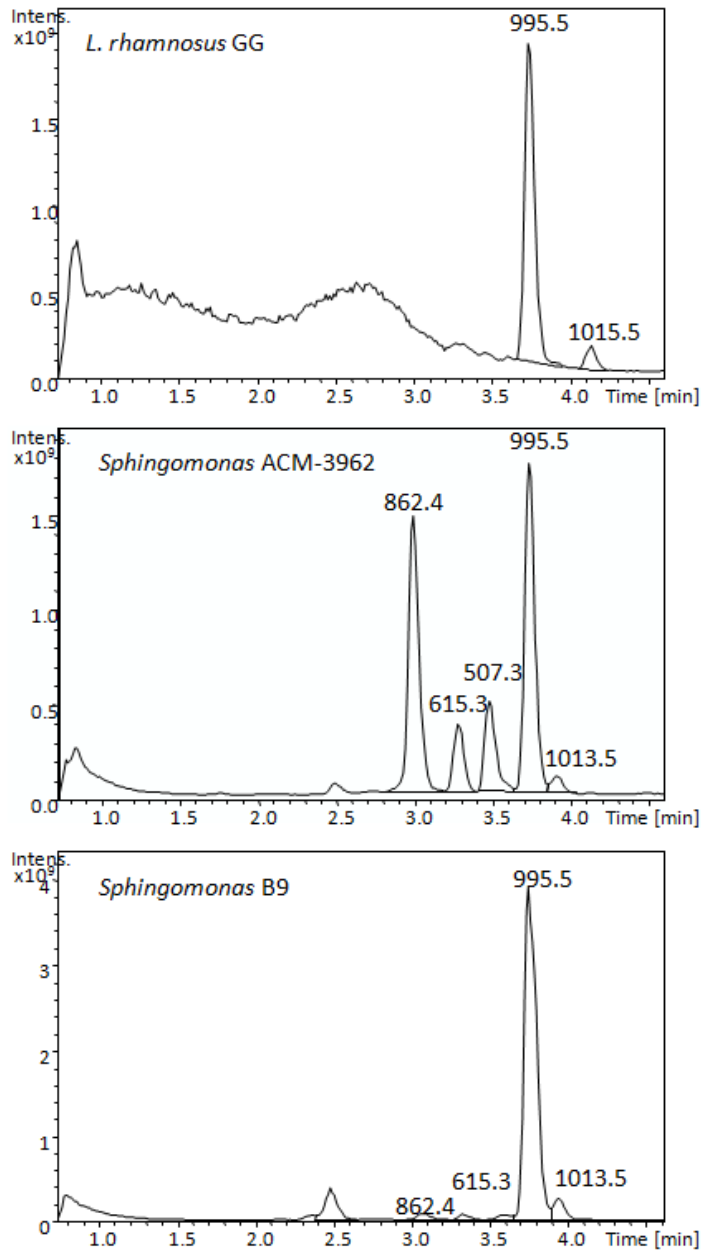
**Figure 4.13.** Comparison of MC-LR removal by *L. rhamnosus* GG, *Spingomonas* ACM-3962 and B9. HPLC chromatograms of bacteria incubated with MC-LR for 24 h at 28 °C (*Spingomonas* ACM-3962 and B9) or 37 °C (*L. rhamnosus* GG).

**Table 4.6.** Degradation products found for *Spingomonas* strains

Toxin or degradation product and fragment ion	<i>m/z</i>
MC-LR [M+H] <sup>+</sup>	995.5
Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH [M+H] <sup>+</sup>	1013.5
Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH – NH <sub>2</sub> – PhCH <sub>2</sub> CHOME	862
Adda-Glu-Mdha-Ala-OH [M+H] <sup>+</sup>	615
Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH [M+2H] <sup>2+</sup>	507

HCT Ultra Ion Trap LC-MS analysis





**Figure 4.14.** Comparison of MC-LR removal by *L. rhamnosus* GG, *Sphingomonas* ACM-3962 and B9. LC-MS chromatograms of bacteria incubated with MC-LR for 24 h at 28 °C (*Sphingomonas* ACM-3962 and B9) or 37 °C (*L. rhamnosus* GG), showing degradation products (listed in Table 4.6).

## 5. GENERAL DISCUSSION

The removal of cyanobacterial toxins by probiotic bacteria, both commercial strains of lactic acid bacteria and bifidobacteria, and natural strains isolated from the Indonesian dadih fermented milk, was investigated in this thesis. The main points of investigation were different factors influencing the toxin-removal capacities of the probiotic bacteria and identification of the mechanism of toxin-removal.

Five strains of commercial probiotic bacteria (*L. rhamnosus* strains GG and LC-705, *B. longum* 46, and *B. lactis* strains 420 and Bb12) were shown to be the most efficient in microcystin-removal in the initial screening study (I) and were chosen for further experiments (II-IV). Different physiological conditions potentially affecting the removal efficiency were investigated. These included the effect of pH, temperature, toxin concentration, bacterial cell density and cell viability (I and II). The cell viability and the impact of glucose a source of energy was then studied more in detail (III). Furthermore, the location and mechanism of microcystin removal was investigated by studying a possible extracellular enzymatic degradation of microcystins and the degradation pathway was compared to other microcystin-degrading bacteria (IV).

The facts that supported a mechanism involving active toxin elimination instead of physical binding in studies I and II were the slow removal process, the enhanced removal percentage at optimal growth temperature, the requirement of probiotic bacterial viability and the low recovery of eliminated microcystin from bacterial cells. As non-viable bacteria did not have the same ability to remove microcystins as viable bacteria, metabolic activity or elimination was suggested as a plausible mechanism for microcystin removal. A comparison with the previously reported aflatoxin and heavy metal removal suggested that the removal mechanism of cyanotoxins differs from the other contaminants. Heavy metals have been shown to be removed within minutes of incubation (Halttunen *et al.*, 2007a) and the AFB<sub>1</sub>-removal has also been reported to be rapid (El-Nezami *et al.*, 1998a; Peltonen *et al.*, 2001). The rapid removal process as well as the efficacy of non-viable bacteria suggested that the removal mechanism of heavy metals and AFB<sub>1</sub> is physical binding of the contaminants to the probiotic bacterial cells.

The removal of one microcystin variant, MC-LR, by different probiotic strains, both commercial strains and natural strains isolated from the fermented milk product dadih, was investigated under different conditions, with different temperatures and bacterial cell densities in the absence or presence of glucose, to find the optimal conditions for toxin removal (III). Bacterial viability was assessed by traditional plate counting as well as flow cytometry and microscopy. The results of this study showed that natural isolated strains from dadih, an Indonesian traditional fermented product, were able to remove toxins efficiently up to 48 h, while the removal efficiencies of the commercial probiotic strains started to decrease after 24 h of incubation. This could be explained by the different viability properties of the bacterial strains observed during incubation, which could affect their removal capacities. For the dadih strains, a clear improvement in the removal

ability was observed by the addition of glucose as a nutrient source to the solution, but the increase was not so marked for the other strains.

The results further demonstrate that both viability and metabolic activity play important roles in the removal of microcystins. Supplementation of glucose provides energy to the bacteria, and thereby, the microcystin-removal efficiencies also increase (III). During incubation, the bacterial strains consume glucose and as a consequence produce lactic acid, which in turn causes cell stress as a result of the decrease in pH. In the studies reported in this thesis, the bacterial cells removed MC-LR from solution faster in the presence of glucose but cell death also occurred earlier. Differences between the bacterial strains could be observed both in their microcystin-removal properties and in the viability of the bacteria during incubation.

The results reveal that cell viability, bacterial cell density, cell culturability, and metabolic activity play important roles in the removal of MC-LR (III). As a consequence of active metabolism, the viable cells become exhausted and stressed. Consequently, besides resulting in high removal ability, supplementation of glucose also resulted in decreased viability. In response to lack of energy or stress conditions, many bacteria are able to convert into a viable but non-culturable condition (VBNC). 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 (Rollins and Colwell, 1986; Colwell, 2000; Lowder *et al.*, 2000; Pruzzo *et al.*, 2002). In other words, they enter an active but non-culturable state. This may be potentially reversed by provision of nutritionally rich culture media and optimal growth conditions (Yamamoto, 2000; Oliver, 2005). The results of this study suggest that the metabolic activity of the bacteria is maintained functional in the cells, which can be detected by the enhanced removal of MC-LR at 37 °C in the presence of glucose. Furthermore, the microscopic images support the fact that glucose enhances the metabolic activity of the cells, and thereby, the cells become stressed and cell death occurs earlier.

Different bacterial strains, other than lactobacilli or bifidobacteria, have been shown to be able to degrade microcystins enzymatically. Bacterial degradation of microcystins has previously been reported for naturally occurring bacterial strains (Cousins *et al.*, 1996; Park *et al.*, 2001; Harada *et al.*, 2004; Kato *et al.*, 2007). Bourne *et al.* (1996) described an enzymatic pathway for the bacterial degradation of toxic cyclic peptides mediated by hydrolytic enzymes of *Sphingomonas* species. The degradation products were identified as linearized MC-LR and further degradation to a tetrapeptide. The probiotic strains studied in this research work could possibly utilise a similar kind of mechanism for MC-LR removal, which was investigated in study IV. The GRAS status of probiotic lactic acid bacteria would give them an advantage for usage in microcystin-removal.

Previous studies with *Sphingomonas* strains (Bourne *et al.*, 2001; Ishii *et al.*, 2004) have indicated that the key enzyme involved in microcystin degradation is present in the periplasmic space. The findings of this thesis indicate that the process of microcystin-removal in the presence of probiotic bacteria differs from the known mechanism of biodegradation that occurs in the cells of

*Sphingomonas*. The key enzyme involved in microcystin-degradation in *Sphingomonas* strains, 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).

Based on the results of study IV it can be concluded that no activity against MC-LR is present in the cytosolic fraction of the investigated probiotic strains, which excludes the involvement of intracellular proteases in the process of MC-LR removal. Additionally, the studies with <sup>14</sup>C-radiolabelled microcystins showed that MC-LR is not transported into the cells of probiotic bacteria during the incubation with bacterial cells but stayed in the supernatant during the whole incubation. Furthermore, cytosolic bacterial cell extracts were not able to remove MC-LR. 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.

Consequently, the participation of cell-envelope proteinases in microcystin removal was investigated. A large increase of enzymatic activity was observed after addition of glucose to the solution during incubation. 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. The proteolytic system present in all species of lactic acid bacteria was therefore the natural candidate for research regarding enzymatic removal of toxins by the probiotic bacteria (IV). It is composed of extracellularly located proteinases involved in the initial cleavage of casein to peptides, peptidases that hydrolyze the large peptides thus formed into smaller peptides, and amino-acids and peptide transport systems involved in the cellular uptake of the hydrolytic products (Law and Haandrikman, 1997). Simultaneous analysis of the enzymatic activity and the rate of MC-LR removal indicated that these parameters are closely related and both decreased with time.

In summary, the results presented in this thesis have for the first time provided evidence that probiotic bacteria may efficiently remove cyanobacterial toxins from aqueous solutions. The correlation between the activity of cell-envelope proteinases and the decrease of MC-LR concentration suggests that enzymes are involved in microcystin removal. The findings strongly support the theory that enzymatic degradation of microcystin occurs when the toxin is incubated with cell suspensions of probiotic bacteria.

Lactic acid bacteria and bifidobacteria interact with toxin producing microbes and some toxins as well as viruses and pathogens present in food and water. The main future objective would therefore be to understand probiotic health effects and to relate the mechanisms and actions to future potential of specific probiotic bacteria in decontamination of foods and water. The complex interaction of microbes with other members of the gut microbiota and the host also forms the basis for assessing interactions between the probiotic bacteria and toxins, as well as other contaminants. The studies of this thesis have focused on the removal of cyanobacterial toxins, but to assess the usefulness of probiotics as biological decontaminants, also interactions with

potentially pathogenic bacteria, fungal toxins and viruses should be taken into account to be able to define future applications in human health promotion. The use of probiotics as a personal defence mechanism against cyanotoxins could be a potential area of use. Probiotic dietary supplements could possibly be used as a personal defence against cyanotoxins in the gastrointestinal tract and to reduce the health risks caused by microcystins in drinking water.

Previous reports have showed that biological degradation of cyanotoxins may be a feasible method of water treatment. By the results obtained in this thesis, probiotic bacteria show potential in biological decontamination of cyanotoxin-containing water. Biological decontamination of drinking water by probiotic bacteria could therefore be another potential target of use. The probiotic bacterial strains or possible enzymes identified in the removal process could be used in a bioremediation process to remove toxins from drinking water. Bacteria immobilised on a filter might be a useful approach for the removal of contaminants such as heavy metals and mycotoxins, for which non-viable bacteria have been shown to be efficient removers. On the other hand, for efficient removal of cyanotoxins, metabolically active viable bacteria are needed, which could be a challenge for the potential filter-approach. In addition, the optimal removal temperature of 37 °C, the high cell density needed, the relatively long treatment time as well as the requirement of a bacterial nutrient source could be a challenge for an efficient water treatment process. The re-use of bacterial cells in the treatment process should further be assessed. The acids secreted in the fermentative metabolism of carbohydrates by probiotics should also be taken into account, as acidification of water could be a possible outcome. Instead, other technologies of biological decontamination could be considered. Recombinant technologies using potential purified enzymes identified in the removal process of probiotic bacteria could be a future approach for efficient cyanotoxin removal.

In conclusion, the development of new treatment technologies by low-cost probiotic bacteria and specific probiotic combinations that would be able to remove or inactivate both mycotoxins, cyanotoxins, heavy metals, viruses and pathogenic bacteria is an important aspect to consider in the future. These kinds of probiotic combinations could be used for decontamination of both water and food and could provide new options for the removal of the contaminants mentioned.

## 6. CONCLUSIONS

Toxic cyanobacteria have been reported in lakes and reservoirs around the world. 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 majority of cyanotoxin biodegradation studies have focused on bacteria isolated from water sources exposed to microcystin-containing blooms. The results of this thesis present a new type of bacteria, specific probiotic bacterial strains, to be efficient in cyanotoxin removal. Probiotic bacteria have several advantages in comparison with the previously reported microcystin-degrading bacteria, as they have been classified as food grade, safe bacteria by EFSA (2010). Therefore probiotic bacteria can safely be included in both food and water, and can also safely be used in food technology. Furthermore, the beneficial health effects of probiotic bacteria give them an advantage for the use in different applications.

The first aim of this study was to screen a number of specific strains of probiotic lactobacilli and bifidobacteria for their capacity to remove microcystins from water solutions and to evaluate their potential for water decontamination (I). Several strains were shown to be efficient in toxin removal and different physiological conditions, including the effect of pH, temperature, toxin concentration, bacterial cell density and cell viability, optimal for removal were investigated (II). The removal of several different cyanotoxins was also studied (II). The third specific aim was to further investigate the role of bacterial viability and how it could be affected by different factors to achieve optimal toxin removal (III). The fourth aim of the study was to identify bacterial removal mechanisms of one of the cyanotoxins studied, the hepatotoxin MC-LR (IV). This was studied by comparison of the degradation pathway of previously identified microcystin-degrading bacteria with the probiotic strain, and by investigating the possible location for toxin degradation. An extracellular enzymatic degradation of microcystins by probiotic bacteria was tested and it was suggested that extracellularly located cell-envelope proteinases appear to be involved in the decomposition of MC-LR. In particular, a correlation between proteolytic activity and MC-LR removal was found.

The main human exposure route of microcystins is considered to be the oral route. Eighty percent of microcystin exposure has been estimated to occur through drinking water (Kuiper-Goodman *et al.*, 1999). The removal of microcystins in drinking water is, therefore, of great importance and probiotic bacteria show promising results in this respect. They could consequently have applications both in biological decontamination of microcystin-containing water and as a personal defence against microcystins in the gastrointestinal tract. There is a high demand for effective and low-cost approaches for removing cyanotoxins from potable water due to the significant health risk and inadequate access to safe drinking water. Bacterial bioremediation is a promising and potentially effective means for removal of cyanotoxins (Bourne *et al.*, 1996; Ho *et al.*, 2007) and probiotic bacterial strains may contribute to treatment strategies.

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