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Aflatoxin Binding by Probiotics
Experimental Studies on Intestinal Aflatoxin Transport, Metabolism and Toxicity

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium ML1, Medistudia building, University of Kuopio, on Saturday 31st March 2007, at 12 noon

School of Public Health and Clinical Nutrition, Clinical Nutrition and Food and Health Research Centre University of Kuopio
ISBN 978-951-27-0741-6 (PDF)
ISSN 1235-0303

Abstract

Aflatoxin B₁ (AFB₁) is a food contaminant with detrimental impact on human and animal health. Intervention approaches focus on pre- and post-harvest measures to reduce AFB₁ levels in crops or on the individual level to modulate bioactivation and excretion of AFB₁ or reduce its bioavailability. Probiotic bacteria have been identified as a potential means to reduce availability of AFB₁ as well as other food contaminants. In this study we used both in vitro and in vivo approaches to study the interplay between probiotic bacteria and AFB₁ in the intestinal environment. Initially, the binding ability of several probiotics was explored in vitro. Intestinal mucus was found to compete with AFB₁ binding sites on the surface of bacteria. This should be taken into consideration when choosing a probiotic for AFB₁ binding, as was also evident from our studies in the duodenal loop of chicks, where different probiotics with similar binding capacity in vitro, had different effects on AFB₁ absorption from the intestine. As a subsequent step, rats were dosed orally with AFB₁ and probiotics and fecal excretion of AFB₁ was significantly but transiently increased by probiotic dosing, possibly reflecting a reduction in absorption of AFB₁ from the intestinal lumen. Furthermore, AFB₁ induced hepatotoxicity was slightly reduced and weight loss was alleviated in rats dosed with probiotics. To study whether the AFB₁ binding by the probiotic bacteria has an effect on its transport and toxicity to the intestinal tissue, Caco-2 cells were incubated with AFB₁ and direct toxic effects on epithelial integrity and genotoxic effects were observed in the presence and absence of the bacteria. When the probiotic bacteria were added, AFB₁ toxicity could be reduced. In conclusion, these studies clearly show the protective effects of probiotic bacteria against AFB₁ induced intestinal and systemic toxicity via binding AFB₁ and reducing its transport in different test systems.

National Library of Medicine Classification: QW 125.5.P7, QW 142.5.A8, QW 630, WI 402
Medical Subject Headings: Aflatoxin B1/metabolism; Aflatoxin B1/toxicity; Caco-2 Cells; Chickens; Duodenum; Feces; Intestinal Absorption; Intestines/metabolism; Lactobacillus; Mucus/microbiology; Propionibacterium; Probiotics; Rats; Urine
“No guts no glory”
Acknowledgements

This work was carried out in the Department of Clinical Nutrition and the Food and Health Research Centre at the University of Kuopio. I would like to express my gratitude to the staff of both facilities, for providing the productive and enjoyable work environment. I would like to express my special thank you to the people below:

To Hannu Mykkänen, my principle supervisor, for always having an open door and time for discussion.

To my supervisor, Hani E-Nezami, for never-ending professional and private counseling, encouragement and a positive attitude towards life. You taught me to take things with a smile.

To Risto Juvonen for teaching me the eye for details and for his repeated effort to make my work more chemical, more toxicological.

To Arthur Ouwehand and Seppo Salminen, for the opportunity to visit the Functional Foods Forum in Turku and for practical supervision during my time there and afterwards.

To Paul Turner and Chris Wild for supporting my wish to work in the Molecular Epidemiology Unit at the University of Leeds.

To Matti Viluksela and the staff of the Animal Unit in the National Public Health Institute for their help to plan and carry out the animal work.

To Barry Goldin and Raimo Pohjanvirta for kindly reviewing this thesis, and giving constructive and beneficial suggestions.

To all the people I have worked in the lab with: Nektaria, Otto, Kaisu, Heidi, Karina, Rianne, Quoc, Sanna, Clare, Jo, Anne, Kay, Satu and many others.

To my dear friends, colleagues, office and travel mates, fellow students, sauna and party company Nektaria and Ursi, for sharing all these countless experiences. Thank you for your company!

To all my Finnish friends, especially Virpi and Otto, for being there from the moment I arrived to the day I leave.

To all my foreign friends, who have passed through Kuopio with me, especially Quoc, Ferdinand, Jakub, Tatjana, Irina, Kaja and many more. Thank you for all the mökkis and parties.

To my family, my parents and sisters, for supporting my idea of living abroad and for always coming to visit.

To Iain, for being the reason and the motivation for everything.
For financial support of this work, I would like to thank the University of Kuopio, the Finnish Graduate School on Applied Bioscience: Bioengineering, Food & Nutrition, Environment, The Finnish Society of Nutrition Research, The Juho Vainio Research Foundation and The Orion Pharma Research Foundation.

Kuopio, March 2007

[Signature]

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Abbreviations

AFB\textsubscript{1}  Aflatoxin B\textsubscript{1}
AFB\textsubscript{1}-GSH  Aflatoxin B\textsubscript{1}-Glutathione conjugate
AFB\textsubscript{2}  Aflatoxin B\textsubscript{2}
AFG\textsubscript{1}  Aflatoxin G\textsubscript{1}
AFG\textsubscript{2}  Aflatoxin G\textsubscript{2}
AFL  Aflatoxicol
AFM\textsubscript{1}  Aflatoxin M\textsubscript{1}
AFM\textsubscript{2}  Aflatoxin M\textsubscript{2}
AFP\textsubscript{1}  Aflatoxin P\textsubscript{1}
AFQ\textsubscript{1}  Aflatoxin Q\textsubscript{1}
ALT  Alanine Transaminase
ATCC  American Tissue Culture Collection
CFU  Colony forming unit
CYP  Cytochrome P450
DMEM  Dulbecco’s Modified Eagle’s Medium
DMSO  Dimethylsulfoxide
EDTA  Ethylenediaminetetraacetic acid
GG  \textit{Lactobacillus rhamnosus} strain GG
HCA  Heterocyclic Amines
HPLC  High Performance Liquid Chromatography
HSCAS  Hydrated sodium calcium aminosilicate
i.p.  Intra peritoneal
IAC  Immunoaffinity column
IARC  International Agency for Research on Cancer
LAB  Lactic acid bacteria
LC-705  \textit{Lactobacillus rhamnosus} strain LC-705
LD\textsubscript{50}  Lethal dose, 50%
NADPH  Nicotinamide adenine dinucleotide phosphate
p.o.  Per oral (per os)
P\textsubscript{e}  Permeability coefficient
PJS  \textit{Propionibacterium freudenreichii ssp. Shermanii} JS
TER  Transepithelial resistance
\textit{1,25(OH)\textsubscript{2}D\textsubscript{3}}  1α,25-dihydroxyvitamin D\textsubscript{3}
List of original publications

This dissertation is based on the following publications, referred to in the text by their Roman numerals (I – IV):


IV. Gratz S., Wu Q. K., El-Nezami H., Juvonen R. O., Mykkänen H., Turner P. C. *Lactobacillus rhamnosus* strain GG reduces aflatoxin B₁ transport, metabolism and toxicity in Caco-2 cells. Submitted to Applied and Environmental Microbiology.

Furthermore some unpublished data are presented.
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1 Introduction

Food is the fuel of life, and we are all concerned about the quality and safety of our food. Harmful components in plant derived foods can be either produced by the plant itself, or are contaminants deriving from manmade sources or from microorganisms. Among these microorganisms, toxin producing fungi are ubiquitous in the environment and can invade our crops and produce toxic secondary metabolites known as mycotoxins. Worldwide, millions of tons of crops are destroyed every year due to fungal growth and spoilage, to reduce human exposure to mycotoxins. Technologies are available to minimize fungal growth and contamination during harvest, processing and storing of crops, but these methods are only available in developed countries, resulting in a divided prevalence of mycotoxin exposure. Low level mycotoxin exposure occurs in parts of the world where food is available in higher quality and variety, whereas high level exposure causes acute disease which may result in death and is prevalent in areas where populations depend on a single staple food commodity.

Aflatoxins are a group of mycotoxins, commonly contaminating maize and groundnuts, and are categorized as class 1 A human carcinogens by the International Agency for Research on Cancer (IARC, 2002). Low level chronic aflatoxin exposure is linked to the development of “occult” conditions such as impaired growth and immune function and chronic diseases such as liver cancer in areas where the aflatoxin producing Aspergillus fungi is prevalent. It is therefore of major interest, to prevent formation of aflatoxins in the first place, or to reduce its bioavailability from foods to prevent harmful effects.

Microorganisms, especially bacteria, have been studied for their potential to either degrade mycotoxins or reduce their bioavailability. Among these bacteria, probiotic lactic acid bacteria have been identified as a safe means to reduce availability of aflatoxins in vitro. Furthermore, probiotic bacteria exert a number of other beneficial health effects, which make them even more suitable additives to food and feed.
In this study, the potential of probiotic bacteria to bind aflatoxin to their surface and thereafter reduce aflatoxin uptake and harmful effects was investigated. Both *in vitro* and *in vivo* experiments were conducted to mimic conditions inside the intestinal tract to evaluate the potential of probiotic bacteria to interfere with processes of absorption and metabolism of AFB₁.
2 Review of the literature

2.1 Intestine

The role of the intestinal tract in digestion and absorption of nutrients and xenobiotics is of fundamental importance in the research fields of nutrition, pharmacology and toxicology. Absorption of chemicals (nutrients, xenobiotics) is greatly influenced by two factors, firstly the intestinal content (gut lumen) with its diverse and complex bacterial ecosystem together with the mixture of ingested material (i.e. food) and digestive juices and secondly the intestinal epithelium (gut wall) with its absorptive and metabolic capacity (Ilett et al., 1990).

The intestinal microbiota form a symbiotic interaction with the host, and provide a range of metabolic activities, which can affect the host in a beneficial or harmful way (Schiffrin and Blum, 2002). The secretion of digestive juices, mucus and bicarbonate determine the organism’s ability to digest and absorb the ingested materials. The quality of ingested material will also affect its digestibility and its effect on the intestinal mucosa. Many food contaminants and drugs are known to damage the intestinal epithelia, and therefore affect the absorptive capacity and well being.

2.1.1 Brief description of anatomical and physiological features

The intestinal mucosa is composed of three layers, the muscularis mucosa (the deepest layer), the lamina propria (the in-between layer of connective tissue) and the mucosal epithelium, a continuous sheet of epithelial cells, one cell thick, lining villi and crypts (Henry, 1982). The mucosal mucosa consists of two sublayers of longitudinal and circular muscle and is involved in the contractile processes of peristalsis in the intestinal tract. The lamina propria, also referred to as submucosa, is composed of extracellular matrix and provides stability to the epithelial cell layer. Embedded are lymph and blood vessels, smooth muscle cells, nerves and a variety of immune competent cells such as plasma cells, lymphocytes and macrophages, all part of the gut associated lymphoid tissue (GALT) (Henry, 1982). The lamina propria also expands into villi, supporting the folds of
the intestinal epithelium structurally, which are most abundant in the upper intestinal tract. The composition is illustrated in Figure 1.

**Figure 1**: Schematic picture of the microanatomy of the digestive tube. Adapted from http://www.adam.com

The most important kinds of epithelial cells tightly lining the surface of the intestinal tract are absorptive enterocytes and secretive goblet cells on the villi and undifferentiated, proliferative cells in the crypts. The absorptive cells (enterocytes) are tall columnar cells of polar shape, with microvilli expanding from their apical surface into the intestinal lumen (Doherty and Charman, 2002). Goblet cells lie scattered in between enterocytes, and are responsible for secretion of mucus glycoproteins, which form a protective mucus layer all throughout the intestinal tract (Henry, 1982).

### 2.1.2 Intestinal absorption and metabolism of xenobiotics

The surface of the proximal intestinal tract is the optimal site of absorption of chemicals. Two major modes of absorption are described for the uptake of chemicals from the lumen into the systemic circulation: (a) passive permeability down a concentration gradient (which is most common for xenobiotic absorption) or (b) carrier-mediated uptake which can happen either facilitatively (not energy requiring) or actively (energy consuming) (Doherty and Charman, 2002). A third mode of absorption is paracellular passive permeability (c). The carrier or transporter molecules within the intestinal membrane can either be absorption proteins, often specific for the uptake of nutrients, or efflux proteins (d) (such as P-glycoprotein or multi drug resistance protein (MRP), both members of the ATP-binding cassette superfamily), important for pumping xenobiotics
(drugs, toxins) out of the enterocytes back into the intestinal lumen (Doherty and Charman, 2002). These absorption modes are illustrated in Figure 2.

**Figure 2:** Different pathways for intestinal absorption of a compound. The intestinal absorption of a compound can occur via several pathways: (a) transcellular passive permeability; (b) carrier-mediated transport; and (c) paracellular passive permeability. However, there are also mechanisms that can prevent absorption: (d) intestinal absorption can be limited by P-gp, which is an ATP-dependent efflux transporter; and (e) metabolic enzymes in the cells might metabolize the compound. From (Balimane and Chong, 2005).

Once a chemical has entered the enterocytes, it will be subject to metabolism by the enzymes present (e). For xenobiotics, this basically means phase I and phase II reactions, both aiming at making components more water soluble and easier to excrete (Vermeulen, 1996). One of the most prominent phase I enzyme families is the cytochrome P450 enzymes (CYPs), a superfamily of membrane associated haemoproteins, concentrated in the endoplasmatic reticulum of liver and intestinal cells (Ding and Kaminsky, 2003). Among these, CYP 3A4 is the most important of all human drug metabolizing enzymes, and plays a major role in the intestine, since it is strategically located in high concentrations at the tip of the villus, and is always positioned in close vicinity to the P-glycoprotein within the enterocytes (Lindell et al., 2003). It also plays a role in bioactivation of xenobiotics such as aflatoxin, which will be discussed in detail later. Furthermore, phase II conjugation enzymes are also found in enterocytes in the gut epithelium (Doherty and Charman, 2002).

### 2.1.3 Models to study intestinal metabolism and absorption of chemicals

The intestinal tract has long been studied for its metabolic and absorptive capacity for nutrients and other chemicals. Various models *in vitro* (in the test tube), *ex vivo* (with live
tissue separated from the animal) and in vivo (in the live animal) have been developed, all aiming at improving the proximity of the assay to the “real” situation inside the gut. These methods will be briefly described here.

**In vitro methods**

Processes inside the intestinal lumen contain digestion reactions of nutrients with secreted digestive enzymes, and reactions of nutrients and chemicals with microbiota, colonizing the intestinal tract. In vitro studies allow to mimic the intestinal milieu of the upper intestinal tract by subsequently adding digestive agents such as mucus, digestive enzymes, different pHs (using HCl and bicarbonate) and bile acids into reaction chambers (Versantvoort et al., 2005; Brandon et al., 2006). The lower intestinal tract is modeled by inoculating foods with intestinal microbiota (Aura et al., 2006) in reaction systems called “simulator of the human intestinal microbial ecosystem” (SHIME).

Both in vitro setups are widely used for survival studies of probiotics (Ouwehand et al., 2001). Furthermore, studies on bioaccessibility (i.e. the fraction of the contaminant that is released from the food) of harmful food components have been performed in vitro (Versantvoort et al., 2005). The major disadvantage of this type of study is the lack of absorptive capacity, but recently a combined in vitro digestion/Caco-2 cell culture system was used to study iron bioavailability (i.e. the fraction of an administered dose that reaches the systemic circulation) (Yun et al., 2004).

To study the intestinal metabolism of test compounds, the simplest approach is to separate subcellular fractions of enterocytes (cytosol, microsomes, brushborder fragments, nuclei), usually by a series of centrifugation steps, and then study enzyme activities of these fractions (Peters, 1982; Ilett et al., 1990). Different materials, such as animal tissues, human biopsy samples or cell lines can be used for this approach, and it has allowed the elucidation of many enzymatic pathways in enterocytes and other cell types.

However, absorption can not be studied in this setup. Primary cell culture (where proliferative cells are removed from an animal and cultured for several days) and cell lines (often immortalized cells, provided by tissue culture collections) are widely used today, with Caco-2 cells being most extensively characterized (Balimane and Chong, 2005). Caco-2 cells are derived from human colon carcinoma tissue and differentiate spontaneously into small intestinal epithelia like enterocytes. This feature makes them a strong tool for drug absorption studies, and many clones have been described, which
express different enzyme systems and produce intestinal mucus (Balimane and Chong, 2005).

Tissue pieces or whole organs can be removed from the animal, and also used in culture for a limited amount of time, to allow the study of physiological tissues. Tissue slices and everted intestinal sacs have been used to study transport of nutrients for a long time, and still have some relevance to date in studies of xenobiotics absorption (Hugon et al., 1982; Carrillo et al., 1985; Ilett et al., 1990; Ramos and Hernandez, 1996; Iida et al., 2006; Ohta et al., 2006).

**In situ/ex vivo methods**

In situ perfused gut loops and ex vivo intestinal loops have been used, where the tissue is not removed from the animal, but rather stays in place. Different compartments of the intestine can be separated by ligatures and absorption of chemicals can be studied (Davies, 1980; Bertholon et al., 2006). This kind of study allows testing absorption of components in the small intestine, since the blood flow and physiological conditions inside the loop stay intact.

**In vivo methods**

Physiological models use live animals, dosed with the xenobiotics of interest, and the concentration of the chemical or its metabolites can be determined in the systemic circulation or the target tissue (Ilett et al., 1990). These studies give information about bioavailability, rate of absorption and clearance, for example by studying the xenobiotic or its metabolites in plasma samples. From these measurements, area under the curve values from concentration/time diagrams can be calculated and different xenobiotic preparations or routes of exposure can be compared (Ilett et al., 1990; Hsieh and Wong, 1994). Furthermore, the adverse effects of toxins, systemic or specific organ damage, can be used as markers for internal dose of the compound (e.g. liver damage, body weight of rats).

Given the long list of experimental approaches available to study intestinal absorption and metabolism, the choice of the suitable technique is crucial. A research question has to be clearly defined in order to evaluate, which technique is able to answer it. Clearly in vivo animal experiments have many advantages over in vitro techniques, but ethical issues must be considered when choosing. Current regulations on animal experimentation are based on the principle of the three Rs: Reduction, Replacement and Refinement, which have become the basis for “humane”, ethical animal research (Kolar, 2006). Replacing animal
experiments with other suitable techniques and optimizing animal studies with number of animals and procedures used are most important points to be considered.

Nevertheless, animal experimentation has played a fundamental role in the research field of toxicology, and has provided a vast knowledge about the health risks that xenobiotics such as aflatoxins can pose for animals and humans.

2.2 Aflatoxins

Contamination of feed with *Aspergillus flavus* was first discovered after an outbreak of sudden death among several hundred thousand ducklings and turkeys in the year 1960 and was later named “Turkey X Disease” (Blount, 1961). This finding led to the isolation of a fluorescent compound referred to as aflatoxin as an abbreviation of “*Aspergillus flavus* toxin” (Nesbit et al., 1962). The chemical structures of the six major dietary aflatoxins are shown in Figure 3.

![Chemical structures of major dietary aflatoxins](image)

**Figure 3:** Chemical structures of major dietary aflatoxins namely aflatoxin B₁, G₁ and M₁ with the double bonds in 8-9 positions and aflatoxins B₂, G₂ and M₂ without the double bond.

Chemically, aflatoxins are a group of difuranocoumarin derivatives that show fluorescence under ultraviolet light. According to the color of the fluorescence the aflatoxins are grouped into aflatoxin B₁ and B₂ (AFB₁, AFB₂) for blue, and G₁ and G₂ (AFG₁, AFG₂) for green, where subscripts refer to the chromatographic mobility.
Aflatoxin M₁ and M₂ (AFM₁, AFM₂), known as milk-aflatoxins, are metabolites of AFB₁ and AFB₂ (Carnaghan et al., 1963).

AFB₁ is the most toxic and most prevalent compound, followed by G₁, B₂ and G₂ with decreasing toxicity (Busby, 1984). AFM₁ is frequently detected in dairy products, and its toxicity is comparable to AFB₁ (Busby, 1984). Aflatoxins can be produced by the four toxic species of Aspergillus: A. flavus, A. flavus ssp. parasiticus, A. nomius and A. pseudeotamarii (Pitt, 2000; CAST, 2003) as secondary metabolites. Occurrence of these molds and therefore aflatoxin contamination is limited to warm and humid climates and most frequently detected in Sub-Saharan Africa and Southeast Asia. Food commodities most frequently detected with aflatoxins are corn and corn products, peanuts, dried fruits and dairy products (AFM₁) (IARC, 1993). Daily human aflatoxin exposure varies between countries and was estimated between 4-184 ng/kg body weight in various African countries, 12-2027 ng/kg body weight in Southern China and 7-53 ng/kg body weight in Thailand, as compared to <3 ng/kg body weight in the USA (Hall and Wild, 1994; Williams et al., 2004). Maximum legal concentrations of total aflatoxins in foodstuffs are set in many countries, the EU for example has set 4 μg/kg food intended for direct human consumption (de Koe, 1999). The further part of this literature review will focus on aflatoxin B₁ and its metabolites.

2.2.1 Toxicokinetics of aflatoxin B₁

Route of exposure and absorption

AFB₁ is a common food contaminant and exposure in humans and animals mainly occurs through the oral route. However, inhalation of contaminated grain dust was found to be a major source of AFB₁ exposure in people in special occupational settings (Cullen and Newberne, 1994). Following ingestion, aflatoxin B₁ is efficiently absorbed in the intestinal tract, and the duodenum was found as the major site of absorption (Hsieh and Wong, 1994). Since AFB₁ is a low molecular weight compound, passive diffusion into the enterocyte was suggested as a mechanism of absorption (Kumagai, 1989; Hsieh and Wong, 1994; Fernandez et al., 1997). These findings are further supported by a study showing that AFB₁ transport through a Caco-2 monolayer occurs at similar rates from apical to basolateral side and vice versa (Mata et al., 2004). This study suggests that no efflux pumps or transporters are involved in AFB₁ absorption or extrusion, although another study (Loe et al., 1997) found a multidrug resistance protein that extruded
aflatoxin-glutathione conjugate (AFB₁-GSH) and low levels of unmetabolized AFB₁ from cells. Following respiratory exposure, AFB₁ might appear in the blood more rapidly than after oral exposure, but after 4 hours the plasma concentration-time plots did not differ between the two routes of exposure (Coulombe and Sharma, 1985). Following absorption, first pass metabolism of AFB₁ already occurs in the intestinal and respiratory epithelium, although the impact of these metabolic sites is still to be evaluated. From the site of absorption, AFB₁ enters the blood stream and is transported to the liver, the major site of metabolism.

**Metabolism**

Metabolism of several dietary aflatoxins follows similar pathways, but with respect to occurrence and toxic effect, this review focuses on aflatoxin B₁. Metabolism of xenobiotics including AFB₁ can be divided into three phases, bioactivation (phase I), conjugation (phase II) and deconjugation (phase III), all of which can occur directly at the site of absorption, in the blood, after entering the liver as the main metabolizing organ, or in several extra-hepatic tissues (Vermeulen, 1996). Aflatoxin B₁ itself is not a potent toxin, and phase I bioactivation is needed to exert toxic effects (Massey et al., 1995). Phase I reactions are mainly oxidation of AFB₁ to hydroxylated metabolites such as aflatoxin M₁, aflatoxin Q₁ and aflatoxin P₁ and to the highly reactive AFB₁-8,9-epoxide (Eaton and Gallagher, 1994; Eaton et al., 1994). This epoxide can occur in two isomers, the endo- and the exo-form, but only the exo-isomer is of relevance in terms of toxicity and carcinogenicity (Massey et al., 1995). The formed epoxide is highly unstable, and will readily bind to biological nucleophils such as nucleic acids (alkylation) to form stable adducts with RNA and DNA (Eaton et al., 1994; Smela et al., 2001). Covalent binding of AFB₁-8,9-epoxide to DNA is known to induce point mutations and DNA strand breaks, and is linked to the carcinogenic effects of AFB₁ exposure. In the presence of water, the epoxide will be rapidly and non-enzymatically hydrolyzed to AFB₁-8,9-dihydrodiol, which is able to form Schiff bases with primary amino groups in lysine residues (Sabbioni et al., 1987). One of the proteins, readily available for AFB₁ adduct formation is serum albumin, forming a stable adduct persisting in the blood circulation of rats for several days (Sabbioni et al., 1987) and humans for several weeks. Therefore, levels of AFB₁-albumin or AFB₁-lysine after proteolytic digestion are widely used as biomarkers of AFB₁ exposure. The mechanism of diol formation and protein adduction is most likely involved in the acute toxic effects of aflatoxin (Eaton et al., 1994). Thus it is possible that aflatoxin could
cause gross damage to cells at the intestinal interface, reducing nutrient uptake, or may specifically target important functional sites such as nutrient transporters or tight junctions. Major metabolic pathways are summarized in Figure 4.

![Figure 4](image-url)


Cytochrome P450 enzymes (CYPs) are known to play the major role in oxidation of AFB1 to the reactive epoxide in many tissues, although lipoxygenases and prostaglandin H synthase, in the presence of arachidonic acid (Battista and Marnett, 1985; Massey et al., 1995), have been shown to have the capacity, in humans, to catalyse this oxidation in extra-hepatic organs.
CYP enzymes are a family of haemoproteins with the capacity for monooxygenase enzymic metabolism of toxic hydrocarbons. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) is required as a cofactor and oxygen is used as a substrate (Vermeulen, 1996). Although predominantly expressed within the liver, CYPs are additionally expressed extra-hepatically within most tissues and especially in the respiratory and intestinal tract, thereby providing intestinal cells in vivo with the capacity to bioactivate aflatoxin (Larsson and Tjalve, 1995).

CYP nomenclature uses Arabic numbers for the family and letters for the subfamily of enzymes. Of the CYP enzymes, CYP 1A2 and the CYP 3A family play a fundamental role in aflatoxin bioactivation (Massey et al., 1995; Gallagher et al., 1996). CYP 3A4 has the capacity to metabolize AFB1 to form the AFB1-exo-8,9-epoxide, whilst the majority of its enzymatic action hydroxylates AFB1 to aflatoxin Q1 (AFQ1), a less toxic metabolite (Ueng et al., 1995). The formation of AFQ1 and AFB1-exo-8,9-epoxide at a ratio of 10:1 was observed in CYP 3A4 complementary DNA (cDNA)-expressing lymphoblastoid microsomal preparations (Gallagher et al., 1996). Conversely, CYP 1A2 metabolism forms the hydroxylated aflatoxin M1 (AFM1) and the AFB1 epoxide in a ratio 1:2.5 in the same system. However, CYP 1A2 was found to produce a mixture of both the endo- and exo-isomer of the epoxide (Guengerich et al., 1996). A recent study suggests that CYP 3A4 contributes to the total hepatic AFB1-epoxide formation with 79% in human liver microsomes, and that CYP 3A5, a polymorphic variant of the gene, also has a significant role in high expressers (Kamdem et al., 2006). The degree of contribution may also depend on AFB1 exposure levels with low levels favoring CYP 1A2 (Hengstler et al., 1999).

Hydroxylated AFB1 metabolites (AFQ1, AFM1, AFP1) are less toxic because they are much poorer substrates for epoxidation. (Cullen and Newberne, 1994). However, AFM1 has also been shown to exert direct toxic properties without metabolic activation, in contrast to AFB1 (Neal et al., 1998) Reduction of the 1-keto group of AFB1 produces the metabolite aflatoxicol (AFL) (Busby, 1984). This reduction is catalyzed by a cytosolic reductase and AFL can be readily oxidized back to AFB1. AFL is not considered a significant AFB1 detoxification product since it has been shown to have comparable carcinogenicity and 70% the mutagenicity of AFB1 (Eaton et al., 1994) and can readily be oxidized back to AFB1. It was therefore suggested to be a “reservoir” for AFB1 in vivo (Hsieh and Wong, 1994).
Phase II metabolism includes conjugation of phase I metabolites with glutathione or glucuronic acid and is considered detoxification to enhance water solubility and excretion (Massey et al., 1995). Epoxide can be conjugated with glutathione with the help of glutathione-S-transferase (Degen and Neumann, 1978; Cullen and Newberne, 1994), an enzyme essential in the reduction and prevention of AFB1 induced carcinogenicity. Conjugates of epoxide and hydroxylated AFB1 metabolites are readily excreted via the bile into the intestinal tract, where they might be subject to bacterial deconjugation as phase III reaction. The metabolism and toxicity of AFB1 have been studied in human cellular systems derived from liver (Knasmüller et al., 2004) or respiratory tract (Van Vleet et al., 2001), but the impact of the intestinal metabolism is still to be investigated.

**Excretion**

The major route of excretion of AFB1 and its metabolites is the biliary pathway, followed by the urinary pathway. In lactating animals, AFM1 and other metabolites are excreted in the milk.

Coulombe and Sharma (1985) found the cumulative excretion of AFB1 radioactivity over 23 days after a single dose (0.6 mg/kg body weight) to be 55% in feces and 15% in urine of rats. In rat bile, AFB1-GSH conjugate was identified as the major metabolite, followed by AFP1 glucuronide (Hsieh and Wong, 1994). Reabsorption of bile-borne metabolites only occurs at high dose levels (0.5-2.5 mg/kg body weight) (Degen and Neumann, 1978), when chloroform extractable, absorbable metabolites were detected, but not at low dose levels (Hsieh and Wong, 1994). Deconjugation of conjugated biliary AFB1 metabolites by intestinal microbiota may occur, but has not been studied extensively. In lambs, AFM1 was detected as the only aflatoxin metabolite found in feces besides dietary AFB1 and AFG2 (Fernandez et al., 1997), and in humans, AFQ1 and AFM1 were detected from fecal samples of AFB1 exposed subjects (Mykkänen et al., 2005). Fecal excretion of AFB1 and metabolites has mainly been assessed either via analyzing contents of bile, or by measuring total fecal radioactivity following administration of radioactive AFB1. Neither method gives definite information about the presence of unabsorbed AFB1 in the fecal material. Given the efficiency of the intestinal epithelium to absorb AFB1, detection of unabsorbed AFB1 is unlikely, but may occur at high dose levels.

In urine, the three major metabolites found in rat and human are AFM1, AFB1-N7-guanine, the degradation product of hepatic AFB1-DNA adducts, and AFP1. The hydroxylated AFB1 metabolites, including AFM1, AFQ1 and the demethylation metabolite
aflatoxin $P_1$ (AFP$_1$), are excreted from the body in urine (Groupman et al., 1985; Hsieh and Wong, 1994). Recently, a 10-fold higher level of AFQ$_1$ has been found in human urine compared to that of AFM$_1$ (Mykkänen et al., 2005).

### 2.2.2 Toxicity and carcinogenicity of aflatoxins

#### Effects on humans

A recent outbreak of aflatoxicosis in Kenya has resulted in 125 deaths among 317 cases of poisoning (CDC, 2004). The case fatality rate was 39%, caused by levels of AFB$_1$ in home-grown maize reaching up to 8 mg/kg maize. Several previous outbreaks of aflatoxicosis have occurred in Africa and India, mostly in adults with poor nutritional status and maize as staple food (IARC, 1993). The clinical picture indicated acute toxic liver injury manifested as jaundice with a mortality rate of 10-60% (Peraica et al., 1999). From these findings it can be concluded that the acute lethal dose for adult humans is in the order of 10-20 mg (Pitt, 2000).

In humans, numerous studies have linked the incidence of primary hepatocellular carcinoma with the intake of aflatoxins, leading to the classification of AFB$_1$ as class 1 human carcinogen by the IARC (IARC, 1993). Areas of high incidence of liver cancer such as China, Taiwan and sub-Saharan Africa, also have the highest prevalence for aflatoxin exposure and hepatitis B virus, leading to the theory that these two hepatocarcinogens act synergistically (Kew, 2003).

Besides the carcinogenic effects, aflatoxins are also implicated with immunomodulatory effects and the occurrence of infectious disease as well as with growth faltering effects in children (Gong et al., 2002; Williams et al., 2004). Epidemiological studies show geographical similarities in the occurrence of aflatoxins in food and kwashiorkor (Peraica et al., 1999).

#### Effects on animals

Toxic and especially carcinogenic effects of aflatoxins have been reported in several different animals, but susceptibility to these toxins varies greatly with sex, age, species and strain within a species (Busby, 1984; CAST, 2003). Experimentally verified LD$_{50}$ values (lethal dose for 50% of animals) for rats for example, vary between 0.75 and 17.9 mg/kg body weight between animals of different age, sex or strain (Busby, 1984). Numerous animal studies have shown that the liver is the main target organ and therefore the main
symptoms of aflatoxin exposure in domestic and laboratory animals are hepatic injuries (Busby, 1984; Robens and Richard, 1992; IARC, 1993).

### Table 1: Acute toxicity of dietary aflatoxins in various species.

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Species (all male)</th>
<th>Age/weight</th>
<th>Route of exposure</th>
<th>LD50 mg/kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>Chicken</td>
<td>21 days</td>
<td>p.o.*</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Mouse (CFW swiss)</td>
<td>30 days (20g)</td>
<td>p.o.</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>Rat (Porton-Wistar)</td>
<td>100-150g</td>
<td>p.o.</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>Rat (Fischer)</td>
<td>200g</td>
<td>p.o.</td>
<td>1.16</td>
</tr>
<tr>
<td>AFG1</td>
<td>Rat (Fischer)</td>
<td>200g</td>
<td>p.o.</td>
<td>1.5-2.0</td>
</tr>
<tr>
<td>AFB2</td>
<td>Rat (Fischer)</td>
<td>200g</td>
<td>p.o.</td>
<td>&gt;100x of AFB1</td>
</tr>
<tr>
<td>AFG2</td>
<td>Rat (Fischer)</td>
<td>200g</td>
<td>p.o.</td>
<td>&gt;100x of AFB1</td>
</tr>
<tr>
<td>AFB1</td>
<td>Duck (Pekin)</td>
<td>50g</td>
<td>i.p.*</td>
<td>0.73</td>
</tr>
<tr>
<td>AFG1</td>
<td>Duck (Pekin)</td>
<td>50g</td>
<td>i.p.</td>
<td>1.76</td>
</tr>
<tr>
<td>AFB2</td>
<td>Duck (Pekin)</td>
<td>50g</td>
<td>i.p.</td>
<td>1.18</td>
</tr>
<tr>
<td>AFG2</td>
<td>Duck (Pekin)</td>
<td>50g</td>
<td>i.p.</td>
<td>2.83</td>
</tr>
<tr>
<td>AFM1</td>
<td>Duck (Pekin)</td>
<td>40-50g</td>
<td>p.o.</td>
<td>Similar to AFB1</td>
</tr>
<tr>
<td>AFM2</td>
<td>Duck (Pekin)</td>
<td>40-50g</td>
<td>p.o.</td>
<td>&gt;4x of AFB1</td>
</tr>
</tbody>
</table>

* p.o. per oral, i.p intraperitoneal.

Data obtained from (Busby, 1984; IARC, 1993; Cullen and Newberne, 1994; Roebuck and Maxuitenko, 1994).

Effect of aflatoxicosis on farm animals have been thoroughly studied and reviewed (Robens and Richard, 1992). They report that swine and cattle fed high doses of aflatoxins show liver changes such as centrilobular congestion and hemorrhage or increased prothrombin time, although cattle seem to be less susceptible than swine. Lower aflatoxin doses may lead to milder hepatic injuries and reduced growth rate, especially in young animals (Pier, 1992). Cattle and poultry show economically significant effects like reduced reproductivity and feed efficiency, immunomodulation or reduced milk and egg production, and poultry is reported to be susceptible to aflatoxicosis (Robens and Richard, 1992).

Aflatoxin B1 requires microsomal oxidation to the reactive AFB1-8,9-epoxide to exert its carcinogenic effects. This intermediate reacts with DNA, forming persistent adducts, which induce mutations in somatic cells (Fink-Gremmels, 1999). As for the mechanism of AFB1 induced mutagenicity and carcinogenicity, AFB1-epoxide adducts to DNA, preferably guanine nucleotides, causing point mutations mainly G-C to T-A (94%) or A-T (6%) (Bailey et al., 1996). Depending on the location this mutation occurs, activation of proto-oncogenes or silencing of suppressor genes will cause initiation of the cancer process. Mutations in the p53 tumor suppressor gene at the hot spot of codon 249, are
discussed in the context of AFB\(_1\), but relations between this mutation, AFB\(_1\) and hepatitis B virus infection are not entirely clear (Smela et al., 2001). Following this initial mutation, cytotoxic effects of AFB\(_1\) will further promote cancer development, which leads to the definition of AFB\(_1\) as a "complete carcinogen" (Massey et al., 1995).

Aflatoxin B\(_1\) has been demonstrated to be the most potent liver carcinogen known in different animal species (Pitt, 2000). Several animal studies report the carcinogenicity of aflatoxins in various species. Trout and rat are the most susceptible species, whereas mice seem to be relatively resistant to aflatoxin induced carcinogenicity due to effective glutathione conjugation capability (Dragan and Pitiot, 1994). Busby and Wogan (Busby, 1984) summarized that primary liver tumors induced by oral aflatoxin B\(_1\) have been found in fish (trout, salmon), birds (duck), rodents (rat, mouse, hamster), ferrets and monkeys (Rhesus monkey, African green monkey). Some animal studies show tumors in several other organs like colon, glandular stomach and kidneys (Busby, 1984; Dragan and Pitiot, 1994).

2.2.3 Methods to control the aflatoxin problem

A recent outbreak of aflatoxicosis in May 2004 in Kenya (CDC, 2004) has reminded us that the aflatoxin problem, although being known for decades, has not been solved. Due to the increasing number of reports on the toxic nature of aflatoxins, there is a need to control the aflatoxin levels in food and feed. Methods of control can be classified in two categories: (1) prevention of mold contamination and growth and (2) detoxification of contaminated products (Riley and Norred, 1999; Mishra and Das, 2003).

The prevention of mold growth can be achieved by pre- or post-harvest strategies. Potential pre-harvest approaches include measures to reduce crop stress and associated fungal colonization, the use of non-aflatoxigenic strains of *Aspergillus flavus* to out-compete the toxigenic strains, and genetic engineering to produce more resistant crops (Williams et al., 2004). However, these methods are mainly available for farmers in developed countries, leaving developing countries without solutions. Post-harvest methods aim at dry and mold free crops via removing damaged or infected products or using antifungal agents (Riley and Norred, 1999). A recent post-harvest intervention, incorporating a package of activities focused on improved crop drying and storage techniques, successfully demonstrated a greater than 50% reduction in aflatoxin-albumin adducts in a rural population in West Africa, naturally exposed to AFB\(_1\) through diet.
(Turner et al., 2005). In the outbreak of aflatoxicosis in Kenya, improper home storage of maize was identified as a risk factor for jaundice (CDC, 2004). Detoxification refers only to post-harvest treatments designed to remove or destroy (decontaminate) the toxin and therefore reduce the toxic effects (detoxify) of the contaminated product. It can include physical, chemical or biological methods (Scott, 1998).

Among physical treatments, cleaning and sorting of grain or peanuts as well as segregation are promising but incomplete methods (Riley and Norred, 1999). The use of adsorbents added to animal feed is one approach, using hydrated sodium calcium aluminosilicate (HSCAS) to reduce the bioavailability of aflatoxins (Phillips et al., 2002; CAST, 2003). Recently, HSCAS have also been demonstrated to be safe for humans (Wang et al., 2005), which would allow the use of this technique for products intended for human consumption. Within all chemical treatments, only ammoniation is in extensive commercial use for cottonseed meal, peanut meal or sunflower meal. Ammonium degrades aflatoxins to nontoxic metabolites, but it can cause slight changes in the nutritional quality of feed (Phillips, 1994). Sodium bisulfite, a common food additive, is a promising detoxification treatment and also ozonization has been found to degrade aflatoxin in corn at minimal cost and minimal nutrient destruction (CAST, 2003).

Microorganisms like yeasts, molds and bacteria have been tested on their ability to modify or inactivate aflatoxins. *Flavobacterium aurantiacum* has been shown to remove aflatoxin B$_1$ from liquid media (Phillips, 1994) and is used in peanut processing as biodegrader (Diarra et al., 2005).

However, each of these approaches is limited in applicability to certain products and complete elimination of contamination is not achieved. Therefore, additional interventions at the individual level are being sought. The proof of principle in chemoprevention (the use of chemicals to try to reduce the risk of, or delay the development or recurrence of, cancer) of aflatoxin toxicity has been demonstrated with oltipraz and chlorophyllin. These compounds modify aflatoxin metabolism and reduce the biologically effective dose (Kensler et al., 2004), but this approach is unlikely to be used in practice as drug therapy is expensive (Williams et al., 2004). More recently the search for chemopreventive agents has focused on natural products, available and inexpensive, that would modulate aflatoxin metabolism (activation and detoxification). Numerous phytochemicals including isothiocyanates and indole-3-carbinol from *Brassica* species (Kensler et al., 2005); (Manson et al., 1998), flavonoids from green tea (Luo et al., 2006) or allicin from garlic (Berges et al., 2004) are being investigated for their potential to inactivate phase I
activation and/or induce phase II detoxification. This might, in the future, lead to the
definition of an “anticancerous diet”. Furthermore, probiotic bacteria may also have a
protective effect against AFB1 toxicity, and the following section will describe this
approach in detail.

2.3 Probiotic bacteria

Probiotics are defined by Fuller (Fuller, 1991) as “live microbial food supplements
which beneficially affect the host either directly or indirectly by improving its intestinal
microbial balance”. A probiotic should meet several criteria: a) being able to exert proven
beneficial effects on the host; b) being non-pathogenic and non-toxic; c) being present as
living cells; d) being able to survive the passage through the gut and resistant against
metabolic enzymes; e) being stable and remain viable through storage (Pathmakanthan et
al., 2000). Many probiotic organisms have their origins in fermented foods, and their
“History of safe use” in human consumption allows the status of generally recognized as
safe (GRAS) (Donohue, 2004).

When discussing probiotic bacteria, the term Lactic acid bacteria will often be used.
Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive, non-sporing, non-
respiring cocci or rods, producing lactic acid as the major end-product of carbohydrate
fermentation and comprise strains from the genera *Aerococcus*, *Alliococcus*,
*Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactohaera*, *Leuconostoc*,
*Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*
(Axelsson, 2004). As probiotics, only strains from the genus *Lactobacillus* (e.g.
*L. acidophilus*, *L. casei*, *L. bulgaricus*, *L. reuteri*, *L. plantarum*, *L. rhamnosus*) are used.

Furthermore, the genus *Bifidobacterium*, often considered in the same context as
LAB, is phylogenetically unrelated and has a unique mode of sugar fermentation. Among
them, strains like *B. bifidum*, *B. longum*, *B. breve*, *B. infantis* and *B. animalis* are
important probiotics (Goldin, 1998; Salminen et al., 2004).

The genus *Propionibacterium* is of special interest in food production with several
strains being used as dairy starters to produce flavor compounds, carbon dioxide and
propionic acid, a preserving agent (Ouwehand, 2004). In recent studies *Propionibacterium
freudenreichii* has also been shown to have probiotic properties (Ouwehand, 2004).
Furthermore, some other bacteria, yeast and molds are described as probiotics (Goldin,
1998).
2.3.1 Beneficial health effects of probiotic bacteria

In recent years many studies aimed at explaining the mechanisms through which probiotics beneficially affect human health. Salminen and coworkers (Salminen et al., 1996) report that strengthening of the gut mucosal barrier in healthy subjects could be such a mechanism and interaction between probiotics and epithelial cells is needed. In order to increase the time of this interaction, probiotics must adhere to the intestinal surface, as a first step to its colonization. Adhesion abilities of beneficial bacteria have been widely studied, using different intestinal substrates such as intestinal mucus of various sources, intestinal epithelial cells of animal or human origin and from immortalized cell lines (Tuomola, 1999). 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; Tuomola et al., 2000; Rojas et al., 2002). It is not yet clear, whether different sites are used for adhesion to different substrates (mucus or intestinal cell), or by different strains of bacteria, or whether all binding sites are involved simultaneously (Rojas and Conway, 1996; Tuomola et al., 2000).

One basic mechanism of probiotic action is to modify the normal gut microflora. The normal human microbiota is a complex ecosystem, with over 500 different bacterial species present in our large intestine and increasing in number from proximal to distal parts of the intestinal tract (10^3 CFU/g stomach content to 10^{12} CFU/g colon content) with the predominant genera changing from Gram-positive aerobes to Gram-negative anaerobes (Salminen et al., 1995; Casas and Dobrogosz, 2000). Although different strains of the genus Lactobacillus (L. acidophilus, L. fermentum, L. plantarum) can be isolated from feces of 78% of subjects (Conway, 1995), their numbers are generally low and their importance for a normal function of the gastrointestinal tract is not clearly known. Bifidobacteria are literally absent in the human adult microflora, even though they form the predominant genus of the neonate flora (Conway, 1995). It is therefore believed that by adding these bacteria as probiotics to the diet, the normal flora 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 literature available on the potential and proven health effects of probiotics is vast, and only a brief summary will be presented here.
Generally, health benefits of probiotics are studied by using single bacterial strains, and therefore the proven effects can only be defined for one specific strain (Salminen et al., 2004). To date, the strongest scientifically established evidence for beneficial effect and clinical use of probiotics in humans is in the management of diarrheal diseases (Salminen et al., 2004), including antibiotic-associated diarrhea and infective diarrhea such as Rotavirus diarrhea (Casas and Dobrogosz, 2000) or Traveler’s diarrhea in both adults and children (Pathmakanthan et al., 2000; Boyle et al., 2006).

Furthermore, the effect of LAB to alleviate symptoms of lactose intolerance and of food allergies in infants (Salminen et al., 1996; Goldin, 1998; Salminen et al., 2004) is well established. Numerous studies have focused on immunomodulation by probiotic treatment, and many potential benefits are discussed [increased serum IgA by viable Lactobacillus rhamnosus GG (Salminen et al., 1996), stimulated non-specific intestinal immune reactions with Bifidobacteria (Casas and Dobrogosz, 2000), prevention of the development of atopic disease (Kalliomäki et al., 2001)]. These findings are promising for the future since the incidence of allergies and atopic reactions is increasing.

The potential of probiotics to decrease serum cholesterol has been investigated and Pathmakanthan et al. (Casas and Dobrogosz, 2000) conclude that strong evidence of hypocholesterolaemic effects is available in vitro and in vivo in animals, possibly via deconjugating bile and increasing fecal excretion of bile acids (Lichtenstein and Goldin, 2004).

**Antimutagenic and anticarcinogenic effects of probiotics and LAB**

Colon cancer is a common health problem in the Western world and its occurrence is closely related to the diet. A high intake of fruits and vegetables as well as fermented dairy products may reduce the risk of cancer (Cummings, 1997). The low incidence of colon cancer in Northern Europe may be linked to a significantly higher intake of dairy products and cereals in the normal diet (Rafter, 1995).

Many different ways of action are proposed for LAB to reduce the risk of intestinal cancer. They include influencing the mutagenicity of the intestinal content on one hand or altering the composition and metabolic activity of the intestinal microbiota and therefore reducing bacterial β-glucuronidase, β-glucosidase, nitroreductase and urease on the other hand (McBain and Macfarlane, 1998; Rowland and Gangolli, 1999; Hirayama and Rafter, 2000). Salminen and coworkers (Salminen et al., 1996) summarize several studies
investigating antimutagenic and anticarcinogenic effects of probiotics and report that *L. acidophilus* significantly reduces the mutagenicity of feces and urine of healthy subjects. This was confirmed by Hosoda and coworkers (Hosoda et al., 1996) who found that *L. acidophilus* LA-2 administered in fermented milk reduced fecal mutagenicity in male volunteers and that the excretion of *Lactobacilli* and *Bifidobacteria* in the feces was increased in most of the subjects during the intake of LA-2 fermented milk. More specifically, a reduction in fecal levels of β-glucuronidase were detected after probiotic administration (*L. acidophilus*, *L. rhamnosus* GG and *L. casei* Shirota, several strains of *Bifidobacterium*) in colon cancer patients (Salminen et al., 1996) and in healthy subjects (Casas and Dobrogosz, 2000).

Carcinogen binding has been postulated as a possible mechanism of anticarcinogenicity of probiotics, and is widely studied *in vitro* and *in vivo*. Most commonly studied are the carcinogenic heterocyclic amines (HCA) [including 2-amino3-methyl-3H-imidazo(4,5-f)quinoline (IQ), 2-amino3,4-dimethylimidazo(4,5-f)quinoline (MeIQ), 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx) and 5-phenyl-2-amino-1-methylimidazo(4,5-f)pyridine], tryptophane derivatives [including 3-amino-1,4-dimethyl-5-H-pyrido(4,3-b)indole (Trp-P-1) and 3-amino-1-Methyl-5-H-pyrido(4,3-b)indole (Trp-P-2)], and benzo(a)pyrene (B(a)P).

Rowland and Gangolli (Rowland and Gangolli, 1999) review several studies about LAB and their ability to bind food carcinogens and conclude that there is some experimental evidence in rats that administered LAB decrease the amount of administered carcinogens reaching the blood.

*In vitro* evidence shows good binding ability of various probiotic bacteria strains (*B. longum*, *L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus* 2038 and *Streptococcus thermophilus* 1131) for a range of food carcinogens (PhIP, MeIQ, MeIQx, Trp-P-2, Trp-P-1 and B(a)P) (Bolognani et al., 1997; Terahara et al., 1998). From *in vitro* results, it is also evident that pH dependent differences occur in the carcinogen binding ability of each strain (Bolognani et al., 1997; Terahara et al., 1998).

*In vivo* evidence however, is limited to a much smaller range of bacterial strains and chemicals. *B. longum* and *L. acidophilus* did not reduce mutagenicity of heterocyclic amines *in vivo* in a host-mediated assay or reduced carcinogen absorption into tissues when administered to mice (Bolognani et al., 1997). Accordingly, only *Streptococcus thermophilus* 1131 inhibited the absorption of Trp-P-1, but not of MeIQx and
*L. delbrueckii* ssp. *bulgaricus* 2038 did not inhibit the absorption of either carcinogen *in vivo* in rats (Terahara et al., 1998).

In a more recent study, Zsivkovits and colleagues (Zsivkovits et al., 2003) tested the DNA protective effect of LAB against heterocyclic amines *in vivo* in rats. They administered *L. bulgaricus* 291, *S. thermophilus* F4 or V3 and *B. longum* BB536 to rats before or at the same time as chicken or beef mix, prepared from fried meats, and found substantial protection against DNA damage in colonic and hepatic tissues caused by beef mix with all probiotics used. They concluded that this strong reduction can be partly explained by bacterial carcinogen binding, and suggest also indirect protective effects. The same research group published a review, stressing the importance of carcinogen binding by LAB (Knasmüller et al., 2001). On the other hand, a recent review discussing possible anticarcinogenic effects of probiotics suggested that carcinogen binding might only play a minor role (Commame et al., 2005).

### 2.3.2 Aflatoxin binding by probiotics and LAB

Fermentation of food has been used as a method of preservation for centuries, and LAB are reported to reduce mold growth and aflatoxin production (Mokoena et al., 2006). It has been considered that the inhibition of aflatoxin biosynthesis is due to lactic acid or lactic acid metabolites, which are heat-stable and low-molecular weight compounds (Gourama and Bullerman, 1995). Furthermore, systemic beneficial effects of probiotics, as discussed in the previous section, will also play a role in reducing the adverse effect of aflatoxins in animals and humans. However, this section will focus more on the direct interaction between the bacterium and the aflatoxin molecule.

Several bacterial strains, of food or human origin, have been tested for their ability to bind aflatoxins and other mycotoxins to their surface (El-Nezami et al., 2002a; El-Nezami et al., 2002b; Styriak and Conkova, 2002). El-Nezami and colleagues (El-Nezami et al., 1998a) found that gram-positive bacteria (five strains of *Lactobacillus* and one *Propionibacterium*) were more efficient in removing aflatoxin from liquid medium than gram-negative *E. coli*. Among the five strains of *Lactobacillus*, *L. rhamnosus* strain GG (GG) and strain LC-705 (LC-705), appeared to be most efficient binders for aflatoxin B1, removing approximately 80% of AFB1 from liquid media within 0 hours of incubation, which implies that the binding is a very rapid process. These two strains were later confirmed as most efficient AFB1 binders among nine stains of *Lactobacillus* (Haskard et
Peltonen and coworkers (Peltonen et al., 2001) also studied a range of *Lactobacilli* and *Bifidobacteria* and found remarkable differences in AFB<sub>1</sub> binding abilities, even in strains very closely related. This strain specific AFB<sub>1</sub> binding ability of certain probiotic bacteria strains correlates well with their potential to reduce AFB<sub>1</sub> mutagenicity in the Ames assay for *Lactobacilli* and *Bifidobacteria* cultured in MRS broth (Lankaputhra and Shah, 1998) or *Lactobacilli* cultured in milk (Hosoda et al., 1997).

Besides the specificity of the bacterial strain, also the bacterial concentration influences the AFB<sub>1</sub> removal. Different minimum concentrations have been reported such as 5 x 10<sup>9</sup> CFU/ml of either *L. acidophilus* or *B. longum* to remove only 13% of the AFB<sub>1</sub> within one hour (Bolognani et al., 1997) or 2 x 10<sup>9</sup> CFU/ml of *Lactobacilli* and *Propionibacterium* to remove 50% of free AFB<sub>1</sub> but higher binding occurred at 10<sup>10</sup> CFU/ml (El-Nezami et al., 1998a).

When the bacteria are subjected to various chemical and physical treatments, their ability to remove AFB<sub>1</sub> can be increased significantly. Autoclaved cells of *L. casei* remove significantly more AFB<sub>1</sub> from phosphate buffered saline (PBS) compared to viable bacteria (Thyagaraja and Hosono, 1994). Heat treatment (boiling for 1 hour) and acid treatment also significantly enhanced AFB<sub>1</sub> binding by GG and LC-705 (El-Nezami et al., 1998a, b). Peltonen and colleagues compared the binding ability of various strains of viable and heat-treated *Bifidobacteria* and found that the viable bacteria bound 4-56% while heat-treated bacteria bound 12-82% of the AFB<sub>1</sub> (Peltonen et al., 2001). In contrast, Lankaputra and coworkers found that viable bacteria bound more dietary mutagens (including AFB<sub>1</sub>) than heat-treated bacteria (Lankaputhra and Shah, 1998).

As heat-treated bacteria are often more efficient to remove AFB<sub>1</sub> than viable cells, metabolic degradation cannot be the mechanism responsible for AFB<sub>1</sub> removal. It seems to be more likely that the toxin is bound to the bacterial surface. The aflatoxin-bacteria-complex was therefore studied to test the stability of the interaction, and toxin release was observed after washing the AFB<sub>1</sub>-bacteria complex with water (Oatley et al., 2000; Lee et al., 2003). This finding led to the conclusion that the bacteria only reversibly bound AFB<sub>1</sub>. However, one study reported that bound AFB<sub>1</sub> was only extractable from heat-killed bacteria, but not from viable ones (Lankaputhra and Shah, 1998).

To elucidate the nature of potential AFB<sub>1</sub> binding sites on the surface of *Lactobacilli*, bacteria were subjected to various chemical, physical and enzymatic treatments, and cell wall polysaccharides and peptidoglycans were found to be responsible for the binding of AFB<sub>1</sub> to the surface of GG and LC-705 (Haskard et al., 2000; Haskard et al., 2001). This
was further confirmed by Lahtinen and coworkers (Lahtinen et al., 2004) who studied different cell wall components (exopolysaccharides, cell wall isolates and peptidoglycans) of GG and concluded that peptidoglycans are the most likely binding sites for AFB$_1$. Heat treatment may denature proteins and lead to the formation of maillard-products while acid treatment may break glycosidic linkages in polysaccharides and amine linkages in peptides and proteins and therefore increase the pore size in the peptidoglycan layer of the bacterial surface (Haskard et al., 2001). This process could allow the binding of AFB$_1$ to the cell wall and would explain the increased binding ability of heat- and acid-killed bacteria compared to viable bacteria.

Besides these in vitro studies the binding ability of GG, LC-705 and PJS for AFB$_1$ was tested ex vivo in the intestinal lumen of chicks (El-Nezami et al., 2000a). Authors report that GG removed 54%, LC-705 removed 44% and PJS removed 36% of the AFB$_1$ from the soluble fraction of the luminal fluid within one minute. These results imply that bacterial AFB$_1$ binding appears under physiological conditions in the animal and that this could be a way to reduce the bioavailability of aflatoxin in the organism.

In a pilot clinical study the effect of a mixture of LC-705 and PJS on the AFB$_1$ levels in human feces samples was investigated (El-Nezami et al., 2000b). A significant reduction in fecal aflatoxin levels was found in subjects after receiving the probiotic mixture for two weeks compared to subjects receiving a placebo. Authors expected to find higher levels of AFB$_1$ in the feces of the test group as a result of binding the toxin by the bacteria and reducing the uptake into the body. They suggest that the time of sampling (fecal samples were taken one week after commencing the probiotic intervention) may have allowed the major part of AFB$_1$ in the body to be already excreted in the faeces.

To confirm that the reduction of the AFB$_1$ levels in the feces were not a result of accumulation of AFB$_1$ inside the intestinal tract, adhesion capability of the AFB$_1$-probiotic-complex needs to be studied. Kankaanpää and coworkers report that the Caco-2 cell adhesion properties of GG were reduced when the bacteria first bound AFB$_1$ to their surface (Kankaanpää et al., 2000). Similarly, AFB$_1$ binding by Lactobacillus casei reduced its subsequent adhesion to HT29 intestinal cells (Hwang et al., 2005). These results are important, because the adhesion of the probiotic to the epithelium of the intestine is the first step in its colonization of the gastrointestinal tract. Therefore, the adhesion of the AFB$_1$-probiotic-complex would increase the time the gastrointestinal tract is exposed to AFB$_1$. 

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This literature review summarized the strong evidence that some specific probiotic bacteria bind AFB, *in vitro*, but only limited information is available on binding under physiological conditions in the gut *in vivo*. 
3 Aims

The aims of the work presented in this thesis were to study the binding of aflatoxin B$_1$ by probiotic bacteria under physiological conditions present in the gut and to evaluate whether this binding would have an impact on the transport and absorption of aflatoxin B$_1$ and its toxic effects. For this purpose, a series of \textit{in vitro} and \textit{ex vivo} AFB$_1$ binding studies, a single dose study in rats \textit{in vivo} and cell culture experiments were conducted. Consequently, the specific aims of this work were to:

- Study the \textit{ex vivo} binding of AFB$_1$ by probiotic bacteria in duodenal loops of chicks (I), and assess the impact of intestinal mucus on AFB$_1$ binding by probiotics \textit{in vitro} (II).
- Assess AFB$_1$ binding by probiotic bacteria and its impact on absorption and toxicity \textit{in vivo} in rats (III).
- Investigate the impact of AFB$_1$ binding by probiotic bacteria on the intestinal AFB$_1$ uptake and toxicity in an intestinal cell culture model (IV).
4 Experimental

4.1 Bacterial strains

The bacterial strains used were *Lactobacillus rhamnosus* strain GG (GG, ATCC 53013), *Lactobacillus rhamnosus* LC-705 (LC-705, DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* JS (JS, DSM 7067) and a mixture of the two later (LC-705+JS). All were obtained from Valio Ltd. (Helsinki, Finland) as a lyophilized powder and stored at -80°C. Bacterial counts were initially determined by flow cytometry using a Coulter Electronics EPICS Elite ESP cytometer (Beckman Coulter, Inc. Fullerton, CA, USA) equipped with an air cooled 488 nm argon-ion laser at 15 mV. Total bacterial counts were enumerated using the fluorescent emission from SYTO9 (LIVE/DEAD® BacLight™ bacterial viability kit, L-7012, Molecular Probes, OR, USA) at 3.34 µM per 10^6-10^7 bacteria. A 525 nm bandpass filter was used to collect the emission for both strains, and Fluoresbrite® Beads (2.0 µm, Polysciences Inc., PA, USA) were used as an internal calibration. Later on, bacterial counts were estimated using a spectrophotometer and adjusting the optical density at 600 nm (A=0.5 corresponds to approximately 1x10^8 CFU/ml).

Bacterial suspensions were prepared by weighing lyophilized powder and washing once with phosphate buffered saline (PBS, pH 7.4), centrifuging (3 000xg for 7 min, 4°C) and resuspending in PBS (I, II, III), giving final bacterial concentrations of 1-10 x 10^10 CFU/0.5 ml. For cell culture experiments, lyophilized bacteria were suspended in PBS, heat-treated (boiled for 1 hour) and centrifuged (3 000xg for 7 min, 4°C). After washing once with culture medium (Dulbeco’s Modified Eagle’s Medium, DMEM) containing antibiotics, they were centrifuged again and then suspended in DMEM containing antibiotics to have final concentrations of 1-10 x 10^10 CFU/0.5 ml.

4.2 Aflatoxin standards and HPLC quantification

Crystalline aflatoxins (aflatoxin B1, M1, G2 and aflatoxicol hereafter abbreviated as AFB1, AFM1, AFG2 and AFL) were purchased from Sigma Aldrich, St Louis, MO, USA. Stock solutions were prepared in acetonitrile/benzene (98/2). Methanolic working stocks
were prepared by evaporating the acetonitrile/benzene mixture and reconstituting in methanol. AFB₁ concentrations were determined spectrophotometrically at 363 nm (ε₃₆₃=21 800 M⁻¹ cm⁻¹) and stocks were stored at -20°C. Aqueous work solutions of various concentrations were prepared in PBS (I, II), dimethylsulfoxide (DMSO, III) or cell culture medium (DMEM, IV).

To quantify aflatoxins in various samples reverse-phase HPLC was used (Shimadzu Model LC-10ADvp solvent delivery system, Shimadzu Model SIL-10Advp auto-injector). Samples were injected into an ODS Spheri-5 Brownlee column (220 x 4.6 mm, 5 µm; PerkinElmer Inc., Wellesley, MA, USA) fitted with a C₁₈ guard column (PerkinElmer Inc., Wellesley, MA, USA). Two different mobile phases were used for the analysis of either AFB₁ alone or mixtures of AFB₁ and its metabolites. For AFB₁ analysis (I, II), the mobile phase was water/acetonitrile/methanol (60/30/10) at a flow rate of 1 ml/min. The retention time for AFB₁ was around 8 min. For analysis of the mixtures of AFs (III, IV) mobile phase used was ammonium acetate (20 mM, pH 3.9)/methanol/acetonitrile (70/15/15, v/v/v) at 1.5 ml/min flow rate. The retention times were around 6, 25 and 30 minutes for AFM₁, AFB₁ and AFL, respectively. The aflatoxin peaks were detected by fluorescence detector (RF-10AXL, Shimadzu) at 360 nm (excitation) and 440 nm (emission) and by UV detector at 360 nm and quantified by a Class VP 5.0 software (Shimadzu Koyoto, Japan). The assay was carried out at 40°C oven temperature and with an injection volume of 10 µl.

4.3 In vitro AFB₁ binding by probiotic bacteria (I, II, IV)

Bacterial suspensions (1-10x10¹⁰ CFU/ml either viable or heat-treated) were centrifuged (3 000xg for 7 min, 4°C) and then suspended in aqueous AFB₁ solution (0.1-64 µM) and incubated at 37°C for 1, 5, 10, 15 or 30 min to allow AFB₁ binding. After incubation for up to 30 min, samples were centrifuged, the amount of free AFB₁ in the supernatant was quantified by HPLC and expressed as percentage of AFB₁ added. Additionally, AFB₁ bound to bacterial pellet was extracted twice with 2 ml of chloroform or methanol. Both extracts were combined, evaporated to dryness under nitrogen stream at 40°C and reconstituted in 0.5 ml methanol for HPLC analysis.
4.4 *Ex vivo* binding of AFB\textsubscript{1} by probiotic bacteria (I)

For the *ex vivo* experiment, 80 broiler chicks (age 3 weeks, Vilppulan Hybrid Ltd., Vilppula, Finland) were fasted for one day prior to experiments. Animals were anesthetized with 0.15 ml/100 g body weight of Mebunat\textsuperscript{®} solution (Orion, Finland) i.p. containing 60 mg/ml of sodium pentobarbital. This dose was sufficient to keep the animal under anesthesia for 60 min. A 2-3 cm cross-sectional cut in the lower abdominal region was made and a 5-7 cm long segment of the duodenum around the pancreas was separated by two ligatures. Before tightening the second ligature the test solutions were injected into the loop. One group (AFB\textsubscript{1} group, n=40) received an injection of aflatoxin B\textsubscript{1} (8 \(\mu\)g or 26 nmol/kg body weight in 0.25 ml PBS), and the other group (AFB\textsubscript{1} + LC-705+JS, n=40) received an injection of probiotic mixture LC-705+JS (10\textsuperscript{11} CFU in 0.25 ml PBS) immediately followed by an aflatoxin B\textsubscript{1} injection (8 \(\mu\)g or 26 nmol/kg b.w. in 0.25 ml PBS). Eight chicks from both groups were euthanized at 1, 5, 10, 15 or 30 min after the injection and the duodenal loops were removed from the carcass. Two animals, not receiving any injection, were included as untreated controls. Animal studies were carried out in the animal facilities within the University of Kuopio and were approved by the Animal Ethics Committee of the University of Kuopio.

*Analysis of AFB\textsubscript{1} from intestinal content*

In order to remove all intestinal content, the duodenal loops were rinsed with 25 ml of PBS. Content was centrifuged and supernatant and pellet were extracted and treated as described for *in vitro* experiments. Bacterial AFB\textsubscript{1} binding was calculated by subtracting the amount of AFB\textsubscript{1} in duodenal content of control chicks (no LC-705+JS administered) from amount of AFB\textsubscript{1} in LC-705+JS dosed chicks, and expressed as percentage of the AFB\textsubscript{1} dose.

*Analysis of AFB\textsubscript{1} from duodenal tissue*

The uptake of AFB\textsubscript{1} by the intestinal tissue was also investigated. The duodenal loop was cut into small pieces and homogenized with 10 ml chloroform. The homogenate was filtered, evaporated to dryness and reconstituted in 0.5 ml of methanol for HPLC analysis. Recovery of AFB\textsubscript{1} from biological fluid and tissue was examined by spiking 1.5 \(\mu\)g of AFB\textsubscript{1} into lumen content and duodenal tissue samples and extracting as mentioned above. Values were not corrected for recovery.
4.5 Impact of intestinal mucus on \textit{in vitro} AFB\textsubscript{1} binding by probiotic bacteria (II)

The effect of intestinal mucus, present under physiological conditions in the gut, on aflatoxin binding by probiotic bacteria was tested in the \textit{in vitro} binding assay. Intestinal mucus was collected from three porcine colon samples kindly provided by the Animal Centre of the University of Kuopio. After removing the intestinal content, the tissue was carefully rinsed with PBS and the mucus layer was gently scraped from the mucosa with a rubber spatula. After collection, mucus from three animals was pooled and centrifuged (10 min, 13 000×g, 4°C) to remove cellular debris and bacteria. Prior to use, the protein content of the mucus suspension was determined by a protein assay using a bovine serum albumin standard (Biorad, Espoo, Finland).

For AFB\textsubscript{1} binding experiments, bacteria (GG or LC-705+JS), intestinal mucus and AFB\textsubscript{1} (0.32 - 64.1 µM in 0.5 ml PBS) were mixed either subsequently (forming a bacteria-mucus complex first, II) or simultaneously (I) and incubated for 1 - 30 min and the AFB\textsubscript{1} binding assay was performed as described in section 4.3.

4.6 Impact of probiotic AFB\textsubscript{1} binding on absorption and toxicity of AFB\textsubscript{1} in rats \textit{in vivo} (III)

To assess whether the binding of AFB\textsubscript{1} by probiotics, as observed under \textit{in vitro} conditions, will have an impact on the absorption of AFB\textsubscript{1} from the gastrointestinal tract and on its toxic effects, a single AFB\textsubscript{1} dose was administered to rats pre-dosed with probiotics. Animal experiments were carried out in the animal facilities within the National Public Health Institute (KTL) in Kuopio and were approved by the Animal Ethics Committee of the University of Kuopio.

28 male Han-Wistar rats (age 5 weeks) were provided from the breeding facilities of the University of Kuopio. Rats were kept individually in metabolic cages and maintained on standard powdered feed (R36, Lactamin, Stockholm, Sweden) and water \textit{ad libitum}. They were under a photoperiodic cycle of 12 h light/12 h dark in an air-conditioned room with the mean temperature of 21.0±0.2°C and the relative humidity 50±3%. After an acclimatization period of 3 days, 24 animals were divided into two groups (n=12 rats/group). Group one received the vehicle (PBS) and group two the probiotic strain GG (5x10\textsuperscript{10} CFU/0.5 ml PBS) by oral gavage daily for 6 successive days. Immediately after
the fourth GG dose, animals of both groups received a single oral dose of AFB₁ (1.5 mg or 4.8 μmoles/kg b.w. in 0.5 ml DMSO). Four additional rats served as untreated controls. Body weight was recorded at the beginning of the study (prior to GG treatment), on the day of AFB₁ dosing, and at the end of the study. Urine and feces were collected daily, weighed and stored in -20°C until analysis. At the end of the study rats were anesthetized by CO₂ inhalation and blood samples were taken by cardiac puncture into heparinized Venoject 5 ml blood collection tubes (Terumo Europe N.V., Leuven, Belgium). After centrifugation the plasma samples were stored at -20°C. Alanine transaminase (ALT) activity, a marker for liver injury of AFB₁, was measured in rat plasma using a commercial kit (Konelab, Thermo Electron Corp., Waltham, MA, USA).

4.7 Analysis of biological samples for AFB₁ and its metabolites

Analysis of AFB₁-albumin adducts from rat plasma

The levels of AFB₁-albumin adduct were determined by albumin extraction, digestion and ELISA as previously described (Chapot and Wild, 1991). The detection limit was 5 pg AFB₁-lysine equivalents per mg of albumin (pg/mg). Three positive and one negative control samples were analyzed with each batch of samples. Samples were measured in quadruplicates on two separate days with CVs below 25%.

Analysis of AFB₁ and AFM₁ from fecal samples

Extraction of fecal samples was modified from a method developed in our laboratory (Mykkänen et al., 2005). After collection, fecal samples were weighed and stored in plastic bags at -20°C until analysis. Samples were mixed for 180 seconds (Stomacher 400 Laboratory Blender, GW Berg & Co, Vantaa, Finland) with 2.5 x volume of 0.2 M sodium acetate in 10% NaCl. Aliquots (2 ml) of the mixture were spiked with AFG₂ (18.6 pmol/sample) as internal standard and centrifuged (3000xg, 15 min, 4°C). Pellets were suspended in 4 ml 80% methanol (in 10% NaCl, v/v), vortexed and homogenized thoroughly (MICCRA D-8, ART Labortechnik, Mühlheim, Germany). Following a second centrifugation, the supernatant was reduced to a volume of 1 ml (under N₂-stream, 50°C), diluted with 9 ml of Milli-Q water and aflatoxin residues were isolated using solid phase extraction columns (Strata C18-E 55um, 70A, Phenomenex, Fenno Medical, Vantaa, Finland). Columns were pre-activated with 10 ml methanol followed by 10 ml Milli-Q water. Samples were loaded at a flow rate of 1 ml/min and columns were washed with
5 ml of 5% methanol. Aflatoxin residues were eluted from the columns with 3 ml of acidified methanol (0.5% acetic acid in 50/50 methanol/water) followed by 5 ml of methanol, evaporated to approximately 100 µl volume and diluted with 2 ml of Mili-Q water for Immuno affinity column (IAC) cleanup. IACs (AflaTest, Vicam, Fleurs, Belgium) were washed with 10 ml PBS and 10 ml MQ-water. Samples were loaded, washed with 5 ml PBS and 10 ml MQ-water and eluted with 4 ml of 95% methanol. Cleaned samples were evaporated to dryness under vacuum (SPD1010 SpeedVac® System, Thermo Savant, Waltham, MA, USA), reconstituted in 500 µl methanol and stored at -20°C prior to HPLC analysis.

**Analysis of AFM₁ from urine samples**

After collection, urine samples were weighed, acidified with 0.1 N HCl to pH 5, and centrifuged (3 000xg, 15 min, 4°C). Supernatants were stored at -20°C until analysis. Urine (0.5 ml) was spiked with AFG₂ as internal standard (9.3 pmol/sample) and aflatoxin residues were isolated with solid phase extraction columns and cleaned with IAC as described above for fecal samples. Cleaned samples were evaporated to dryness, reconstituted in 500 µl methanol and stored at -20°C as described above.

**4.8 Quantification of fecal and urinary AF levels**

All fecal and urine samples were spiked with AFG₂ (18.6 and 9.3 pmol per sample, respectively) as an internal standard prior to extraction and analysis. Peak areas of analytes in the fecal and urine samples after HPLC analysis were corrected for day to day variation to a mean AFG₂ peak area, as determined from spiking of negative control fecal and urine samples with AFG₂ and consequent extraction and analysis (spiking of 6 samples per matrix, extraction on two different days, with AFG₂ levels applied in the ‘real’ urine and fecal samples). Quantification of AFB₁ and AFM₁ levels in feces and urine was done by calculation of analyte concentration from AFB₁ and AFM₁ standard curves created in feces and urine. The negative control samples for feces and urine were spiked with AFB₁ (all spikings were done in triplicates; for feces: 5 points with concentrations from 0.32-4.8 µM; for urine: 6 points from 0.16-3.2 µM) and with AFM₁ (feces: 5 points from 0.03-0.46 µM; urine: 6 points from 0.03-0.61 µM), and standard curves were drawn. From the corrected analyte peak areas (correction for AFG₂ internal standard as described above), the concentrations for AFM₁ and AFB₁ in the samples were calculated from the standard
curve equations. Daily and total excretion in feces and urine were calculated based on total volumes of urine and feces. AFM$_1$, AFB$_1$ and AFG$_2$ peak identities in the urine and fecal samples were confirmed via spiking experiments with the respective toxin standards and reanalysis with HPLC.

Figure 5: Representative HPLC chromatograms of samples before (black trace) and after (grey trace) spiking experiments. Panel A shows a rat fecal sample spiked with AFM$_1$, AFG$_2$ and AFB$_1$ standards and Panel B shows a rat urine samples spiked with AFM$_1$ and AFG$_2$. 
4.9 Impact of probiotic binding on AFB\textsubscript{1} uptake and toxicity in Caco-2 cells (IV)

These experiments were carried out to assess transport and toxic effects of AFB\textsubscript{1} in a cell culture model in the absence and presence of probiotics. Additionally, these experiments aimed at evaluating the feasibility of the Caco-2 cell culture model for studying probiotic AFB\textsubscript{1} binding and its impact on its intestinal absorption and toxicity.

4.9.1 Cell line and culture conditions

The human colon cancer cell line Caco-2 (ATCC, HTB-37) was cultivated according to standard procedures in Dulbecco’s Modified Eagle’s Medium (DMEM, Cambrex Bio Science, Verviers, Belgium) with 20% fetal bovine serum (FBS, Invitrogen, Paisley, UK) and 0.2 % antibiotic/antimycotic (20 IU/ml penicillin, 20 µg/ml streptomycin, 0.05 mg/l amphotericin B, Invitrogen, Paisley, UK) and cultures were kept at 37°C with 5% CO\textsubscript{2}. Monolayers were passaged at ~80% confluency with 0.05% trypsin-0.02% EDTA (Invitrogen, Paisley, UK). For experiments, cells were seeded in 12-well plates (9x10\textsuperscript{4} cells/ml) and grown for 21 days until cells differentiated into small intestinal cells containing microvilli.

4.9.2 Induction with 1\alpha,25-dihydroxyvitamin D\textsubscript{3}

The cell line has low expression of a key enzyme, CYP 3A4 that generates the aflatoxin epoxide. This enzyme was induced by pre-incubation with 1\alpha,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}) (Schmiedlin-Ren et al., 1997). DMEM containing 0.25 µM 1,25(OH)\textsubscript{2}D\textsubscript{3} was added onto the cell monolayer on day 21 and left for 2 or 5 days.

The extent of CYP 3A4 induction was assessed by testing AFB\textsubscript{1} bioactivation in microsomes extracted from Caco-2 cells. Cells (10\textsuperscript{5} cells/ml) were seeded for microsomal protein extraction in 75 cm\textsuperscript{2} flasks (Nunc, SLS, Nottingham, UK) in a volume of 25 ml cell culture medium. Undifferentiated cells were grown for 3 days, whilst differentiated cells were grown for 21 days, prior to treatment. Flasks were treated with 0 or 250 nM 1,25(OH)\textsubscript{2}D\textsubscript{3} in 0.05% ethanol for 48 hours. Caco-2 microsomes were isolated by cell lysis, repeated centrifugation and protein quantitation (Biorad, Espoo, Finland). Microsomal protein samples were incubated with aflatoxin, essentially as described earlier.
Pooled rat liver microsomes (Invitrogen, Paisley, UK) were used as a positive control for aflatoxin metabolism. 100 µl incubation buffer [80 mM phosphate buffer (pH 7.4), 75 mM tris-HCl (pH 7.4), 5 mM MgCl₂, 0.39 mM NADP⁺, 6 mM glucose-6-phosphate and 0.37 U of glucose-6-phosphate dehydrogenase] were mixed with AFB₁ (64 µM) and 1 mg microsomal protein and incubated at 37°C for 15 min. The reaction was stopped by addition of 500 µl of ice-cold methanol to each sample. Samples were centrifuged (16 450×g, 15 min, 4°C). The supernatant was retained and the pellet washed with 500 µl ice-cold methanol and centrifuged as above. The combined supernatants were evaporated to dryness (SPD1010 SpeedVac® System, Thermo Savant, Waltham, MA, USA) and the residue reconstituted in 500 µl water/methanol (1/1, v/v) before centrifugation at 2000×g for 5 min at 4°C. The supernatant was subsequently analyzed by HPLC.

4.9.3 Transport of AFB₁ and formation of free metabolites

For transport experiments, cells were grown on Transwell® filter inserts (12 mm diameter, 0.4 µm pore size, Corning Inc., Corning, NY, USA), with media volumes of 0.5 ml in the apical and 1.5 ml in the basolateral chamber. Media was changed three times per week and cells were differentiated for 21 days. Following induction with 1,25(OH)₂D₃, the transport of AFB₁ from the apical to the basolateral side was investigated as described previously (Mata et al., 2004) with modifications. GG suspension (1 or 5x10¹⁰ CFU/ml) and AFB₁ solution in DMEM (150µM) were added to the apical chamber of filters and plates were placed on a plate shaker (300 rpm/min) to prevent the bacteria settling on the monolayer. Aliquots (15 µl) of culture medium were taken from both chambers at 0.5, 1, 24, 48 and 72 hours after AFB₁ addition. Aliquots were centrifuged to remove free cells and bacteria, diluted with Milli-Q water when necessary and injected into HPLC for analysis of AFB₁, AFM₁ and AFL. Furthermore, AFB₁ bound by GG was removed from the bacterial pellet after centrifugation by extracting twice with 0.5 ml methanol, and injected into HPLC. For the transport of AFB₁ through the monolayer, permeability coefficients (Pe) were calculated as Pe = Vd/AxΔ%/Δt where Vd is the volume of the donor compartment, A is the surface area of the monolayer, and Δ%/Δt is the percentage mass transported per second (Mata et al., 2004). The identities of metabolites (AFM₁ and AFL) were confirmed via spiking samples of cell culture medium with respective aflatoxin
standards and reanalyzing by HPLC. Amounts of metabolites were calculated by comparing peak areas of samples with peak areas of standards.

4.9.4 Assessment of AFB<sub>1</sub> induced membrane damage (TER)

Transepithelial resistance (TER) is a measure of membrane integrity, using two electrodes to measure the electrical potential difference (expressed as $\Omega$/cm$^2$) between the apical and basolateral chamber of the two compartment model. Differentiation and AFB<sub>1</sub> induced membrane damage were monitored by changes of transepithelial resistance (EVOM TER-meter, World Precision Instruments, Sarasota, FL, USA). Experiments were carried out as mentioned above, and TER readings were recorded from filters during differentiation, after 1,25(OH)<sub>2</sub>D<sub>3</sub> induction and after addition of GG (1 or 5x10<sup>10</sup> CFU/ml) and AFB<sub>1</sub> (150 $\mu$M). Measurements were always carried out following the addition of fresh culture medium. Following the final measurement, cells were trypsinized and cell viability was assessed using trypan blue exclusion assay. Cell viability was always high (>90% viability).

4.9.5 Assessment of AFB<sub>1</sub> induced DNA damage (Comet assay)

Caco-2 cells were differentiated on 12 well plates for 21 days and induced with 1,25(OH)<sub>2</sub>D<sub>3</sub> for 5 days. Following 24 or 72 hours of incubation with GG (1 or 5x10<sup>10</sup> CFU/ml) and AFB<sub>1</sub> (100 or 200 $\mu$M), the cells were harvested using trypsin-EDTA and viability was assessed with trypan blue staining. Only cells with viability > 90% were used for further assays. DNA damage was assessed using single-cell gel electrophoresis (Comet assay). Single-cell suspensions were prepared in 0.8% low melting point agarose, (in PBS, pH 7.4), placed on a microscope slide pre-coated with 1% agarose and allowed to solidify.Slides were then submerged in detergent solution (2.5 M NaCl, 1 mM EDTA, 10 mM Tris, 10% DMSO and 1% Triton-x, pH 10) for 1 hour to disrupt the cell membranes and washed three times in enzyme buffer (40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA, 0.3 ml 30% bovine serum albumin/500 ml buffer, pH 8) for 5 min before the addition of 50 $\mu$l of Fpg enzyme (formamidopyrimidine [fapy]-DNA glycosylase, diluted in enzyme buffer) or enzyme buffer alone to each slide. Slides were covered with coverslips and incubated at 37°C for 30 min. After removing coverslips, slides were placed in an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH>13) for 40 min, allowing the DNA
to unwind. Subsequently slides were transferred to an electrophoresis chamber at 23V (variable amplitude) for 20 min. After rinsing with neutralizing buffer for 5 min (0.4 M Tris base, pH 7.5), nuclear DNA was stained with ethidium bromide (25 µg/ml). Comets were viewed using fluorescent microscopy and quantified as a percentage of the total DNA in the comet tail using ‘Komet 4.0’ software (Kinetic Imaging Ltd, Merseyside, UK).

4.10 Statistics

SPSS® 11.5 for Windows was used for statistical analysis and data were subjected to either Student’s t-test or Mann-Whitney U-test. Significant differences in the mean values are reported at p ≤ 0.05.
5 Results

5.1 AFB₁ binding characteristics of the probiotics used

In this study two different probiotic preparations were used, *Lactobacillus rhamnosus* strain GG (GG) (II, III and IV) and the probiotic mixture of *Lactobacillus rhamnosus* strain LC-705 and *Propionibacterium freudenreichii* ssp. *Shermanii JS* (LC-705+JS) (I, II). The abilities of these strains to bind AFB₁ *in vitro* as viable or non-viable preparations and at different bacterial counts are summarized in Table 1.

<table>
<thead>
<tr>
<th>Probiotic/number</th>
<th>% AFB₁ bound (±SD)</th>
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<tbody>
<tr>
<td></td>
<td>GG</td>
</tr>
<tr>
<td>Viable</td>
<td>Heat-treated*</td>
</tr>
<tr>
<td>1x10¹⁰ CFU/ml</td>
<td>26.4 (±0.6)</td>
</tr>
<tr>
<td>5x10¹⁰ CFU/ml</td>
<td>60.2 (±1.1)</td>
</tr>
<tr>
<td>10x10¹⁰ CFU/ml</td>
<td>76.3 (±0.5)</td>
</tr>
</tbody>
</table>

*Bacterial suspensions in PBS were boiled for 1 hour.

In this study, both probiotics bound AFB₁ efficiently, although binding percentages for the mixture LC-705+JS were always below the values obtained for GG. Heat treatment enhanced AFB₁ binding ability of both probiotics. However, to perform other beneficial probiotic effects, bacteria have to be administered viable, which explains the use of viable bacteria in most of our studies (I, II, III).

The first part of this thesis work was conducted to assess the binding ability of the probiotic mixture under physiological conditions at a bacterial count high enough to exert sufficient AFB₁ binding (10x10¹⁰ CFU/ml).
5.2 Probiotic AFB₁ binding *ex vivo* and the impact of intestinal mucus 
*on in vitro* binding (I, II)

To assess probiotic AFB₁ binding and its absorption *ex vivo*, AFB₁ was measured in the luminal content and duodenal tissue of chicks injected with a single dose of AFB₁ alone or AFB₁ and probiotic (LC-705+JS). The percentage of AFB₁ retained inside the lumen in the presence of probiotic was significantly (p<0.01) higher at all time points (9 to 39%) compared to the group injected AFB₁ alone (4 to 14%, Figure 6). In the negative controls, no AFB₁ was detected in the duodenal content and tissue.

![Figure 6](image_url)

**Figure 6**: Percentage AFB₁ recovered from duodenal lumen content and duodenal tissue of chicks injected (instilled into duodenal loop) either AFB₁ (4.8 µM) only or AFB₁ and probiotic mixture LC705+JS (10¹¹ CFU/0.5 ml). Values are means of 8 animals ± SD.

Less AFB₁ was detected in duodenal tissues of chicks injected with both probiotics and AFB₁ after 1, 5, 10 and 30 mins when compared to chicks injected with AFB₁ alone (Figure 6). This indicates a 40% reduction in the uptake of AFB₁ by the duodenal tissue after 1 minute when the probiotic mixture was present. From results in Figure 6, the % of AFB₁ bound by bacteria can be estimated by subtracting amounts of AFB₁ present in duodenal lumen in the absence of probiotic mixture from amounts present in the presence of probiotics (% AFB₁ bound = % AFB₁ in content of AFB₁ alone group - % AFB₁ in content of AFB₁ and LC-705+JS group). This suggests that only 25% of the AFB₁ dose were bound by LC-705+JS (10¹¹ CFU/ml) initially after 1 min, and the fraction of AFB₁ bound by the probiotic mixture decreased over time (5% bound after 30 min). In order to explain this lower AFB₁ binding efficiency *ex vivo*, further experiments were carried out *in vitro*, to test the effect of intestinal mucus on the AFB₁ binding by probiotics.
Impact of bacterial mucus binding on AFB₁ binding

The addition of intestinal mucus clearly reduced the binding ability of both probiotics tested (GG and LC-705+JS). However, the effect of mucus was more pronounced for the mixture LC-705+JS (13.6±2.1 vs. 5.3±0.6% of AFB₁ bound in the absence and presence of mucus) than for GG (20.9±0.8 vs. 16.0±1.4% of AFB₁ bound in the absence and presence of mucus) (Figure 7), although GG was able to bind more intestinal mucus (40% of the mucus added) in comparison to LC-705+JS (15%) (II).

Furthermore, a lower amount of mucus was necessary to significantly reduce AFB₁ binding by LC-705+JS compared to GG (II) and mucus decreases AFB₁ binding by probiotic mixture in a concentration dependent manner (Figure 8). Addition of mucus with a protein concentration of 3 mg/ml could not decrease AFB₁ binding significantly at any time point, whereas addition of higher concentrated mucus (6 mg protein/ml) reduced AFB₁ binding by bacteria significantly (p<0.05) after 5, 10, 15 and 30 min incubation time (Figure 8). However, despite the obvious impact of intestinal mucus on AFB₁ binding, the level of binding ex vivo is still lower compared to in vitro results (Figure 8).
5.3 Impact of probiotic AFB₁ binding on absorption and toxicity of AFB₁ in rats in vivo (III)

In order to assess the bacterial AFB₁ binding and its effects in vivo, a single oral AFB₁ dose was given to rats which were dosed with either probiotic suspension (5x10¹⁰ CFU/0.5 ml GG) or vehicle daily for 3 days before and 3 days after AFB₁ dosing.

_AFB₁ binding in vivo_

Bacterial AFB₁ binding was tested in vivo by measuring AFB₁ levels in feces of rats for 3 days following a single oral AFB₁ dose. Fecal AFB₁ levels were highly variable between animals and remained very low in all animals (cumulative fecal excretion over 3 days was 5.3 and 6.8% of the dose for rats in AFB₁ only and AFB₁+GG group, respectively, III). Fecal excretion of AFB₁ was increased by 2.2 fold within the first day after the AFB₁ dose in the group receiving AFB₁ and GG as compared to that receiving AFB₁ alone (35.3±21.3 vs. 15.6±9.8 nmol/24 hour feces, p=0.015), whereas no difference was observed on days 2 and 3 after AFB₁ dosing (Figure 9).
Figure 9: Effect of *Lactobacillus rhamnosus* strain GG (GG) on daily fecal AFB₁ excretion over three days following a single dose of AFB₁ (1.5 mg or 4.8 µmol/kg b.w.). Values represent means±SD (n=12 rats/group).

The percentage of AFB₁ bound by probiotics in feces can be calculated by comparing AFB₁ levels in rats dosed with or without probiotic administration. This shows that additional 19.7 nmoles AFB₁/24 hour feces are excreted in feces due to probiotic binding.

**Intestinal metabolism**

*In vivo* metabolism of AFB₁ in the intestine was assessed by measuring AFM₁ levels in rat fecal samples. The 3 day cumulative excretion of AFM₁ was 5.4% of the dose (Figure 10).
The levels of AFM$_1$ detected in feces one day after AFB$_1$ dosing were significantly higher in the group receiving AFB$_1$ and GG as compared to animals receiving AFB$_1$ only (68.1±26.1 vs. 27.1±22.5 nmol/24 hour feces, p=0.001). On days 2 and 3 following the AFB$_1$ dosing no difference between the groups was observed.

**AFB$_1$ absorption**

AFB$_1$-albumin adducts were detected in the plasma of all rats receiving AFB$_1$, but not in the plasma of the untreated controls. The mean levels of AFB$_1$-albumin adduct in both groups were highly variable and tended to be reduced in the group receiving AFB$_1$ plus GG as compared to that receiving only AFB$_1$ (13.8±4.5 vs. 19.5±10.1 ng/mg albumin, 29% reduction), although this reduction was not statistically significant (p=0.149). No change in urinary AFM$_1$ levels was observed in rats receiving AFB$_1$+GG when compared to rats receiving AFB$_1$ alone (Figure 11).

![Graph](image)

**Figure 11:** Effect of *Lactobacillus rhamnosus* strain GG (GG) on daily urinary AFM$_1$ excretion in rats over three days following a single dose of AFB$_1$ (1.5 mg or 4.8 µmol/kg b.w.). Values represent means±SD (n=12 rats/group).

**Toxic effects on liver function and body weight**

The activity of alanine transaminase (ALT) in plasma, an indicator of liver injury, was highly variable. ALT activity was similar in the control and AFB$_1$ plus GG groups (41.6±18.7, n=4 and 56.4±34.2 U/L, n=9, respectively, p=0.317), while a trend towards increased ALT activity was seen in the group receiving AFB$_1$ alone (103.7±84.9 U/L, n=9, p=0.053) as compared to controls.

Body weight of animals in the beginning of the study was similar in all groups (156.0±23.1 for untreated controls, 155.0±8.0 for the group AFB$_1$ only, and 154.8±7.9 for
During the duration of the study the untreated control animals gained 8.2±2.0 g/day. Prior to AFB₁ dosage the daily body weight gain of the treatments groups were similar (7.0±0.9, p=0.211 and 7.2±1.2 g/day, p=0.332 for AFB₁ alone and AFB₁+GG, respectively) and not different from the body weight gain in untreated controls. During three days following AFB₁ dosing, the reduction in daily body weight gain was significantly more pronounced in animals receiving only AFB₁ (-3.12±2.94 g gain/day) whereas those receiving GG maintained their weight (0.04±2.19 g gain/day, p=0.011, Figure 12).

![Figure 12: Effect of Lactobacillus rhamnosus strain GG (GG) on daily body weight gain before and after a single dose of AFB₁ (1.5 mg or 4.8 µmol/kg b.w.). Values represent means±SD (n=4 rats/group for untreated controls and n=12 rats/group for rats receiving AFB₁ only or AFB₁ and GG).](image)

5.4 Impact of probiotic binding on AFB₁ uptake and toxicity in Caco-2 cells (IV)

In experiments using Caco-2 cells, GG and AFB₁ were added to the apical chamber or a two compartment dish and samples were taken from both apical and basolateral chambers, to assess the effect of AFB₁ binding by GG on AFB₁ transport across the epithelia. From the apical compartment, both free AFB₁ in supernatant and AFB₁ bound by GG from the pellet after centrifugation were determined. Bacteria (1 and 5 x 10¹⁰ CFU/ml) reduced the amount of free AFB₁ in the apical chamber by binding 40.1±5.5 and 61.0±6.0% of AFB₁ added after 1 hour and 18.0±2.8 and 40.9±2.5% after 24 hours, respectively (Figure 13).
Figure 13: Percentage of AFB$_1$ (150 nM) recovered free from culture medium (apical and basolateral) and bound by 0, 1 or 5 x 10$^{10}$ CFU/ml GG (apical). Apical and basolateral culture medium was removed after 1 or 24 hours incubation, centrifuged and supernatant was injected into HPLC. Bacterial pellet (apical) was extracted with methanol to remove AFB$_1$ bound by GG. Values are presented as means of 2 replicates and 2 repeated experiments, error bars refer to standard deviation.

From results of transport experiments, a permeability coefficient can be calculated, to estimate the transport of aflatoxin through the monolayer (Figure 14).

Figure 14: Permeability coefficients (Pe) for AFB$_1$ transport through the Caco-2 monolayer calculated as Pe = Vd/Ax$\Delta$%/A$\Delta$t (Vd = volume of the donor compartment, A = surface area of the monolayer, $\Delta$%/A$\Delta$t = percentage mass transported per second (Mata et al., 2004). Values are presented as means of 2 replicates and 2 repeated experiments, error bars refer to standard deviation.
The permeability of AFB₁ (24.6±3.3x10⁻⁶ cm/sec) was reduced slightly in the presence of 1x10¹⁰ (17.2±6.1x10⁻⁶ cm/sec, p=0.06) and significantly with 5x10¹⁰ CFU/ml (7.0±4.8x10⁻⁶ cm/sec, p=0.005).

**Formation of AFM₁ and AFL by Caco-2 cells**

In the Caco-2 model, we assessed the formation of free AFM₁ and AFL, both metabolites of AFB₁ released into the culture medium of the apical (Figure 15) and basolateral chamber (data not shown).

![Figure 15](image_url)

**Figure 15:** Effect of heat-treated *Lactobacillus rhamnosus* strain GG (GG, 1 and 5x10¹⁰ CFU/ml) on the formation of AFL (panel A) and AFM₁ (panel B) in differentiated Caco-2 cells measured in culture medium of apical chamber at 3 time points (24, 48 and 72 hours) after AFB₁ addition (150μM). Values are presented as means of 2 replicates and 2 repeated experiments, error bars refer to standard deviation.

Without bacteria present, AFL and AFM₁ were detectable in the culture medium of both compartments after 24 hours of incubation of Caco-2 cells with AFB₁ and the levels of both metabolites formed increased further after 48 and 72 hours of incubation.
In the presence of GG, the formation of AFL was reduced in both chambers (apical Figure 15, basolateral data not shown), at both bacterial concentrations and all time points, as could be expected after reduced AFB₁ uptake. Surprisingly, AFM₁ formation was increased in the presence of GG rather than decreased, as would be expected after bacterial binding decreased AFB₁ uptake.

**Intestinal toxicity**

For AFB₁ to perform a toxic effect on Caco-2 cells, induction of CYP 3A4 enzymes is required, for AFB₁-epoxide formation to occur. Therefore, Caco-2 cells were pretreated with 1,25(OH)₂D₃ prior to toxicity assays. TER readings (Transepithelial resistance, a measure for intestinal membrane integrity) increased over 3 weeks of differentiation of the Caco-2 cells, and reached a plateau towards day 21. AFB₁ induced decrease in TER readings was only observed in cells preincubated with 1,25(OH)₂D₃ (Figure 16).

![Figure 16: Effect of heat-treated Lactobacillus rhamnosus strain GG (GG, 1x10¹⁰ CFU/ml) on transepithelial resistance (TER) in differentiated Caco-2 cells (following induction with 1,25(OH)₂D₃ for 48 hours) measured in culture medium of apical and basolateral chamber at 3 time points (0, 24 and 48 hours) after AFB₁ addition (150 μM). Values are presented as means of 2 replicates and 2 repeated experiments, error bars refer to standard deviation.](image)

Incubating 1,25(OH)₂D₃ induced Caco-2 monolayer with 150 μM of AFB₁ significantly decreased the TER ratio (calculated as TERₜₑₒ/TER₀) readings after 24 (0.69±0.04) and 48 hours (0.46±0.02) of incubation (Figure 16). In the presence of probiotic bacteria (1x10¹⁰ CFU/ml), this aflatoxin induced decrease in TER reading is attenuated after 24 hours (0.82±0.02, p=0.002) and 48 hours (0.52±0.01, p=0.04)
incubation, suggesting that bacteria were able to reduce AFB₁ induced toxicity. Higher bacterial numbers and longer incubation times were not suitable for this assay, since bacteria started to influence TER readings, independent of the presence of AFB₁.

As marker of AFB₁ induced DNA damage in the cells, DNA fragmentation was assessed in Caco-2 cells exposed to AFB₁ following induction of CYP 3A4 induction, using the Comet assay to quantify the extent of DNA damage. No DNA damage was seen in the comet assay when Caco-2 cells were incubated either GG, 1,25(OH)₂D₃ or AFB₁ alone (Figure 17).

**Figure 17**: Effect of heat-treated *Lactobacillus rhamnosus* strain GG (GG, 1 and 5x10¹⁰ CFU/ml) on DNA damage in differentiated Caco-2 cells (following induction with 1,25(OH)₂D₃ for 5 days) measured with the Comet assay after AFB₁ addition (200 µM, for 24 or 72 hours). Values are presented as means of 2 replicate slides (50 cells/slide) and 2 repeated experiments, error bars refer to standard deviation. * indicates significant difference (p<0.001) compared to cells incubated without GG.

DNA damage occurred after incubating 1,25(OH)₂D₃ induced Caco-2 with AFB₁ (200 µM, 35.5±11.3 and 63.3±14.0% tail DNA after 24 and 72 hours incubation, Figure 17). A significant (p<0.001) reduction in DNA damage was observed after 24 hours only at the higher GG concentration (5x10¹⁰ CFU GG/ml) (22.8±10.8% tail DNA) and after 72 hours at both GG concentrations (48.1±18.3 and 33.7±14.9% tail DNA for 1 and 5x10¹⁰ CFU GG/ml, Figure 17). Furthermore, AFB₁ at lower concentrations (100 and 150 µM) also caused DNA damage (40.8±17.8 and 52.7±14.7% tail DNA after 72 hours incubation), but GG had no effect on these values (data not shown).
6 Discussion

Probiotic bacteria have been studied intensively for their potential to bind food grade carcinogens, including mycotoxins. A series of studies has identified Lactobacillus rhamnosus strain GG as a very potent AFB₁ binding strain (El-Nezami et al., 1998a, b; Haskard et al., 2001), and it was used as a benchmark in the studies presented here. Furthermore, the probiotic mixture of Lactobacillus rhamnosus strain LC-705 and Propionibacterium freudenreichii ssp. Shermanii JS (LC-705+JS) was used in this study, since it is used by food and feed industry as antifungal agent (Bioprofit™) and was used in a pilot clinical trial in Egypt (El-Nezami et al., 2000a) and a intervention trial in China (El-Nezami et al., 2006), both aimed at reducing naturally occurring AFB₁ exposure in human subjects.

6.1 Probiotic AFB₁ binding ex vivo and the impact of intestinal mucus on in vitro binding (I, II)

The duodenal loop technique in chicks has proven useful for AFB₁ binding and intestinal uptake studies before (El-Nezami et al., 2000a), combining many advantages of providing “real life” intestinal conditions and at the same time having easy access to the intestine to inject test solutions and take samples at various time points. In this earlier study (El-Nezami et al., 2000a), GG, LC-705 and JS bound AFB₁ very efficiently after 60 mins, and the uptake into the duodenal tissue was prevented. In the present study (I), AFB₁ binding probiotic mixture LC-705+JS was between 39% after 1 min and only 4% after 30 mins. Furthermore, AFB₁ absorption into duodenal tissue could only be retarded, but not prevented by probiotic mixture LC-705+JS. These findings led to the question, which factors inside the intestinal lumen of animals could have interfered with AFB₁ binding in such strain-specific manner, reducing binding capacity of the probiotic mixture, but not that of the individual probiotic strains studied before. From the literature it was evident that in vitro assays, e.g. for probiotic adhesion studies, can be modified to include gastric pH, digestive enzymes or intestinal mucus. Previous AFB₁ binding studies clearly showed that addition of HCl did not reduce but rather enhanced bacterial AFB₁ binding by all three probiotic strains used in the present studies (El-Nezami et al., 1998a, b; Haskard et al.,
and that digestive enzymes like lipase (Haskard et al., 2000) or proteases such as pronase E (Haskard et al., 2000), trypsin or α-chymotrypsin (Lahtinen et al., 2004) had no effect on AFB₁ binding by GG.

From intestinal adhesion studies with probiotics it is evident that bacteria have receptors on their surface, enabling them to bind to intestinal epithelial cells and intestinal mucus. Mucin binding proteins have been identified on the surface of probiotic bacteria (Rojas et al., 2002), and heat treatment has been shown to reduce probiotics adhesion abilities (Ouwehand et al., 2000). Many studies have shown these adhesion properties of the probiotic strains used in this study, concluding that GG binds very well to intestinal epithelia and mucus (Kirjavainen et al., 1998; Ouwehand et al., 1999; Kankaanpää et al., 2000; Ouwehand et al., 2000; Morita et al., 2002), and LC-705 and JS show lower binding abilities (Tuomola et al., 2000; Ouwehand 2002). Furthermore, previous AFB₁ binding by probiotics (L. rhamnosus GG and L. casei) have been found to reduce subsequent binding to intestinal cell lines Caco-2 and HT-29 (Kankaanpää et al., 2000; Hwang et al., 2005), suggesting that probiotic adhesion and AFB₁ binding interfere with each other.

Based on these findings, we hypothesized that intestinal mucus, bound to the surface of probiotic bacteria, might impact on the subsequent AFB₁ binding capacity of these probiotics. We therefore adapted the in vitro AFB₁ binding assays to include intestinal mucus. As a first step, we assessed the mucus binding ability of GG and the probiotic mixture LC-705+JS and found that the LC-705+JS removes a smaller amount of mucus as compared to GG, which is in agreement with findings from the adhesion studies. Intestinal mucus was clearly bound rather than degraded by probiotics, since large portions of the bound mucus could be recovered from the bacterial surface by aqueous washing (67% for GG and 31% for LC-705+JS, II) and both Lactobacilli and Propionibacteria have appeared unable to degrade intestinal mucus (Ruseler-van Embden et al., 1995; Ouwehand et al., 2002). We also found that probiotic mucus binding reduced subsequent binding of AFB₁ by GG and LC-705+JS, and vice versa. These findings would suggest direct competition of mucus and AFB₁ for bacterial binding sites, but different bacterial surface components are responsible for mucus binding (Rojas et al., 2002) and AFB₁ binding (Tuomola et al., 2000; Lahtinen et al., 2004). Furthermore, heat treatment of probiotics, known to interfere with protein structures on the bacterial surfaces, has been found to reduce mucus binding (Ouwehand et al., 2000) but at the same time increase
probiotic AFB₁ binding (El-Nezami et al., 1998b). Therefore, the two processes must hinder each other through a different mechanism, e.g. steric hindrance.

The effect of intestinal mucus on bacterial AFB₁ binding was further illustrated by comparing the AFB₁ binding ability of LC-705+JS observed ex vivo in the chick duodenal loops with results from the in vitro assay including intestinal mucus (I). From in vitro experiments, we selected the bacterial number sufficiently large to bind around 60% of the AFB₁ dose. In the presence of intestinal mucus, this was significantly reduced to around 30% AFB₁ binding, but still remained above the values obtained from the ex vivo study (I). This implies that the situation in the intestinal tract of the animal is more complex than can be explained with intestinal mucus and other factors may influence AFB₁ binding.

Strain-specific interference of intestinal mucus with probiotic AFB₁ binding clearly shows the necessity of intestinal mucus to be incorporated into screening assays, aiming at characterizing new candidate probiotics for AFB₁ removal. From studies I and II, we concluded that the LC705+JS mix showing poor AFB₁ binding results in vitro in the presence of intestinal mucus, should not be further used. Consequently, all experiments conducted thereafter (III and IV) were carried out using GG.

6.2 Impact of probiotic AFB₁ binding on absorption and toxicity of AFB₁ in rats in vivo (III)

**Fecal excretion of AFB₁**

Under in vivo conditions, one would expect probiotic bacteria to bind AFB₁ as soon as they interact with each other inside the intestinal tract. Thereafter bacteria should travel through the intestinal tube and be excreted eventually into feces, taking bound AFB₁ with them. Consequently, fecal levels of AFB₁ should allow us to estimate AFB₁ binding occurring in vivo, even though a percentage of binding calculated from results obtained from rat fecal samples lies well below results in vitro or ex vivo. Assessing AFB₁ levels in the fecal sample is difficult and extracting and purifying aflatoxins to an extent that is suitable for further HPLC analysis lead to a poor recovery of AFB₁ from the fecal material. As a result, animal studies on fecal excretion of aflatoxins have used radio-labeled aflatoxin and then measured total excretion of radioactivity in the feces (Coulombe and Sharma, 1985; Hsieh and Wong, 1994). This method however, is not suitable to differentiate between dietary, unabsorbed fecal AFB₁, and hepatic metabolites, then
excreted into the intestinal lumen via the biliary route. A pilot clinical trial conducted in Egypt (El-Nezami et al., 2000a) managed to assess the impact of probiotic supplementation on fecal AFB1 excretion, but showed a reduction in fecal AFB1 level in the presence of probiotics rather than the expected increase. The authors concluded that sample timing was the cause of this effect, since fecal samples were taken one week after starting a daily probiotic intervention. This is in agreement with our in vivo findings, suggesting that probiotic administration only increased fecal excretion of AFB1 within 24 hours of AFB1 dosage, but not at later time points. However, the ultimate goal is to reduce AFB1 bioavailability in the intestinal tract, and an increase in fecal AFB1 excretion in the presence of probiotics at any time point is convincing evidence that this goal can be achieved.

One of the main shortcomings of this in vivo trial was administration of both AFB1 and probiotics via oral gavage. Even though a simple and precise technique, it does not allow any conclusion on probiotic AFB1 binding capacity from contaminated foodstuffs which would be the natural route of exposure.

**AFB1 absorption**

There are two widely used methods to assess AFB1 absorption and exposure in animals and humans. First is to look at the blood circulation of the stable AFB1-albumin adduct (Sabbioni et al., 1987; Hsieh and Wong, 1994; Vinitketkumnuen et al., 1999; Turner et al., 2005) and second is to measure the excretion of metabolites in urine, such as AFM1 and AFB1-N7-guanine (Wogan et al., 1967; Kumagai, 1989; Hsieh and Wong, 1994; Hoogenboom et al., 2001; Walton et al., 2001). In our study (III), the plasma levels of AFB1-albumin adduct were only slightly, but not significantly reduced in rats dosed with probiotic GG. Since the plasma AFB1-albumin adduct has a half-life in rats of only several days (Sabbioni et al., 1987; Vinitketkumnuen et al., 1999), some of the circulating adduct had likely been degraded by the time we measured the AFB1 albumin adduct levels (3 days after dosing). Additionally, we have measured the urinary levels of AFM1 and this did not differ in rats treated either with or without GG.

Findings from the AFB1-albumin adduct and the urinary AFM1 levels may reflect a saturation of the hepatic metabolizing capacity within the dosing regime used (high, toxic, single dose of AFB1). Both biomarkers aim at low detection limits and precise measurements of low aflatoxin levels. However, in a study with dietary levels of AFB1,
they would be useful biomarkers to assess changes in AFB₁ absorption due to probiotics administration.

Formation of AFM₁ and AFL

Besides the binding and absorption, we also investigated the effect of probiotic bacteria on intestinal metabolism and AFB₁ toxicity in rat. Few studies have addressed the role and importance of the intestinal tract in AFB₁ metabolism. Pharmacokinetic studies in rats have identified conjugated metabolites such as AFB₁-glutathione and AFP₁-glucuronidate as major fecal metabolites, and these are clearly excreted into the intestinal lumen via the bile (Hsieh and Wong, 1994). These studies also show that no, or only a very small portion (0.3%) of administered radioactivity can be detected in the chloroform extractable fraction of the rat bile 24 hours after dosing, which would contain free hydroxylated AFB₁ metabolites such as AFM₁ or AFL (Hsieh and Wong, 1994; Degen and Neumann, 1978). These findings suggest that the free aflatoxins found in feces in the present study (4.9 % of the dose, 24 hours after dosing) represent mostly unabsorbed aflatoxin, and possibly some conjugated biliary aflatoxin metabolites that have subsequently been deconjugated by colonic microbiota (Wei et al., 1981). Increased excretion of AFM₁ in the presence of probiotics seems to contradict the expected effect of reduced AFB₁ uptake and hepatic metabolism. Formation of AFM₁ inside the intestinal lumen by microbes can be eliminated, since rat cecal microflora, ruminal microbiota and probiotic bacteria are not able to metabolize AFB₁ to AFM₁ (Wei et al., 1981; Coulombe and Sharma, 1985). However, it can be envisaged that AFB₁ may be metabolized to AFM₁ within the enterocytes of the gastrointestinal system, which expresses CYP 1A2, (Ding and Kaminsky, 2003; Lindell et al., 2003) and that some of these metabolites could diffuse back into the gut lumen. If the gut lumen additionally contains probiotics, re-uptake of AFM₁ by intestinal epithelia may be restricted and the probiotic bound AFM₁ would be excreted via the feces. GG is known to bind AFM₁ equally efficiently as AFB₁ (Pierides et al., 2000; Kabak and Var, 2004), and thus removal of this major toxic metabolite could contribute to the overall increased clearance of aflatoxins from the rat. These data therefore imply that intestinal metabolism of AFB₁ is an important process in the toxicokinetics of AFB₁ and that probiotic bacteria impact upon these processes. However, a recent human study failed to show an impact of probiotics on fecal excretion of any of these metabolites (unpublished data), and further work is needed to fully understand the role of the intestinal epithelium on aflatoxin metabolism.
**AFB$_1$ toxicity**

Liver is the main target organ for AFB$_1$ toxicity and liver-specific enzyme activities in plasma have often been used as toxicity marker. The levels of ALT activity in the control animals and the elevation after the AFB$_1$ dosing in the present study (III) are comparable to values reported in the literature for Wistar rats at a similar AFB$_1$ dose (Lu and Li, 2002). In another study, using Fischer 344 rats, a much stronger elevation in ALT activity due to aflatoxin treatment was observed, confirming the strain specific difference in susceptibility to AFB$_1$ (Maxuitenno et al., 1997). In our study, we observed slightly elevated ALT activity in rat plasma of the AFB$_1$ group (149% increase compared to untreated controls, $p=0.053$), which was significantly associated with AFB$_1$-albumin levels, a marker of aflatoxin intake and hepatic metabolism. In rats treated with GG prior to AFB$_1$, the levels of ALT activity were more similar to controls and were reduced by 46 % compared to the AFB$_1$ only group, though the difference did not reach statistical significance ($p=0.171$). These findings suggest that the probiotic treatment may reduce hepatotoxic effects of a high dose of AFB$_1$ in rats, though high individual variation in liver function and low number of animals may have reduced the power of the study.

Feed intake and body weight gain in rats are reduced by AFB$_1$ in a dose dependent manner at doses ranging from 0.25 to 3 mg/kg body weight (Maxuitenno et al., 1997). In our study, reduction of body weight gain after a single high dose of AFB$_1$ was more pronounced in rats receiving AFB$_1$ only than in animals receiving the probiotic dose additional to AFB$_1$. Probiotic bacteria alone had no effect on body weight gain suggesting that the effect is related to probiotics reducing the amount of free AFB$_1$ within the intestinal tract, and thus lowering toxicity. In children naturally exposed to aflatoxins through diet, aflatoxin biomarker levels have been associated with growth faltering (Gong et al., 2002; Gong et al., 2003; Turner et al., 2003), and one hypothesis is that aflatoxin induced intestinal damage is the cause of this faltering. Since probiotic bacteria may also enhance intestinal barrier integrity (Parvez et al., 2006) they may indirectly protect the intestinal epithelium against AFB$_1$ toxicity.

In conclusion, *in vivo* data suggest that by increasing the excretion of orally dosed aflatoxin via the fecal route, probiotic treatment prevents weight loss and may reduce hepatotoxic effects caused by a high dose of AFB$_1$. However, further studies administering AFB$_1$ repeatedly, mixed into feed and at naturally occurring levels are needed before we fully understand the potential of probiotics to reduce absorption of AFB$_1$ for future use in combating human AFB$_1$ exposure.
6.3 Impact of probiotic binding on AFB$_1$ uptake and toxicity in Caco-2 cells (IV)

The intestinal cell line Caco-2 has been used in mycotoxin absorption studies (Caloni et al., 2002; Mata et al., 2004; Versantvoort et al., 2005; Caloni et al., 2006) as well as in probiotic adhesion studies (El-Nezami et al., 2000a) in the past and has proven a useful tool to reduce the use of experimental animals. In this part of the study we therefore aimed at adapting the Caco-2 cell model for our probiotic AFB$_1$ binding studies, to further assess the impact of probiotic AFB$_1$ binding on its transport, intestinal metabolism and intestinal toxicity. Two compartment cell culture dishes were used to allow measurements of AFB$_1$ transport through the intestinal epithelium and epithelial barrier function. Adjusting the model for AFB$_1$ binding studies appeared to be difficult, since much larger numbers of bacteria are needed (1-10x10$^{10}$ CFU/ml) as compared to probiotic adhesion studies ($10^7$–$10^8$ CFU/ml) (Kirjavainen et al., 1998; Ouwehand et al., 1999; Kankaanpää et al., 2000; Ouwehand et al., 2000; Morita et al., 2002). For this reason, only non-viable bacteria could be used in the present study, since viable bacteria of this number would be metabolically active in the cell culture medium, producing metabolites e.g. lactic acid that would significantly affect the Caco-2 cells viability (unpublished data). However, heat treatment of bacteria reduces their adhesion properties to intestinal surfaces (Ouwehand et al., 2000), and therefore might disable the positive probiotic effect in a host’s intestinal tract.

AFB$_1$ binding in cell culture medium was not different from binding in PBS, and could easily be measured by HPLC. The advantage of the two compartment model is that the effect of tissue uptake on binding can be taken into account, although transport of AFB$_1$ by Caco-2 cells (92% of dose in apical chamber after 30 min) is clearly slower than by the intestinal epithelium (4% of dose in lumen of chicks after 30 min).

From this Caco-2 transport model, permeability coefficients can be easily calculated by comparing AFB$_1$ levels in apical and basolateral chambers. Permeability coefficients (Pe) appear to increase with increasing AFB$_1$ concentrations added, leading to a Pe of 9x10$^{-6}$ cm/sec at 16 nM (Versantvoort et al., 2005), 18x10$^{-6}$ cm/sec at 1µM (Mata et al., 2004) and 25x10$^{-6}$ cm/sec at 160 µM (III). Passive diffusion has been suggested as the mechanism of AFB$_1$ absorption, since transport in both directions, apical to basolateral and basolateral to apical, occurred at similar rates (Mata et al., 2004). In the presence of GG, the Pe was decreased dramatically, suggesting a reduction in AFB$_1$ transport through the
intestinal monolayer. Calculating Pe from the Caco-2 transport model makes it easy to assess transport and to compare results between different studies.

**Formation of AFM\textsubscript{1} and AFL**

The hypothesis of intestinal epithelia cells metabolizing AFB\textsubscript{1}, suggested from our *in vivo* results, is further supported by Caco-2 data, showing that intestinal cells are capable of producing AFM\textsubscript{1} and AFL from AFB\textsubscript{1}. Our results also suggest that the presence of GG (heat-killed though) might even increase the formation of the AFM\textsubscript{1}, since increased amount of AFM\textsubscript{1} were found in both chambers of the transport model. These data certainly need further investigation, clarifying the possible impact of GG on the activity of cytochrome enzymes, responsible for AFM\textsubscript{1} formation. However, AFL formation, known to occur spontaneously under reducing anaerobic conditions or facilitated by cytosolic reductases (Eaton et al., 1994), was clearly reduced in the presence of GG.

**Intestinal toxicity of AFB\textsubscript{1}**

To exert toxic effects, aflatoxin B\textsubscript{1} has to be activated to the highly reactive AFB\textsubscript{1}-8,9-epoxide (Eaton et al., 1994; Massey et al., 1995), which then adducts to proteins and DNA. Caco-2 cells lack the expression of CYP 3A, the key enzyme to facilitate this activation, and 1,25(OH)\textsubscript{2}D\textsubscript{3} has to be used to induce the expression of this enzyme. A decrease in the transepithelial resistance is linked to toxic effects via damaging tight junctions between epithelial cells and therefore disrupting the intestinal barrier function. In our study, we found that a high concentration of AFB\textsubscript{1} (150 μM) reduced TER readings significantly in 1,25(OH)\textsubscript{2}D\textsubscript{3} induced Caco-2 cells without causing gross toxicity. By adding probiotic bacteria this negative effect was attenuated.

Furthermore, we observed significant DNA damage after incubating 1,25(OH)\textsubscript{2}D\textsubscript{3} induced Caco-2 with a high dose of AFB\textsubscript{1}. Only when severe DNA damage occurred, the presence of probiotic bacteria reduced the DNA damage significantly. Compared to studies *in vitro* in rat epithelia (Watzl et al., 1999) and in a liver cell line Hep G2 (Uhl et al., 2000), AFB\textsubscript{1} induced DNA damage in our study was very low, and required a high AFB\textsubscript{1} dose and long incubation time. These findings suggest that induction of the AFB\textsubscript{1} bioactivation was not sufficient to reach CYP 3A levels observed *in vivo*.

Our data clearly demonstrate that GG was able to reduce AFB\textsubscript{1} transport, formation of AFL and DNA damage. However, the use of this cell culture model to study effect of probiotic aflatoxin binding has major limitations because AFB\textsubscript{1} has to be applied in a high
concentration and for a long time (up to 72 hours) for formation of AFB₁ metabolites and damage of DNA in Caco-2 cells to occur. The metabolically modified Caco-2/TC-7 clone (Sambuy et al., 2005), expressing higher levels of CYP 3A enzyme, could be an alternative to further apply this model for probiotic AFB₁ binding studies.
7 Conclusions and future aspects

These studies were conducted to investigate whether probiotic bacteria bind AFB1 under physiological conditions in the gut and whether such binding results in a reduction of AFB1 bioavailability and toxicity. The probiotics used were Lactobacillus rhamnosus strain GG (GG) and a mixture of Lactobacillus rhamnosus LC-705 and Propionibacterium freudenreichii ssp. shermanii JS (LC-705+JS). From the series of experiments conducted, the following conclusions can be drawn:

• Both probiotic preparations were able to bind AFB1 in vitro, but in the intestinal loops of chicks, the probiotic mixture LC705+JS performed less well than anticipated from in vitro results.
• Intestinal mucus was identified as one factor that could influence AFB1 binding by bacteria in a strain specific way, and should therefore be included in in vitro screening assays for new probiotic candidate strains.
• Probiotic GG administration slightly reduced toxic effects of AFB1 in liver and prevented body weight loss of rats via binding AFB1 in the intestinal lumen and increasing its excretion in feces.
• A high concentration of AFB1 was found to be toxic to intestinal cells in a Caco-2 model, and transport and toxic effects were reduced by GG binding of the AFB1 added.

In summary these results show that probiotics, and here especially GG, are able to bind AFB1 under in vivo conditions. However, quantitative assessment of this binding from intestinal content and feces is difficult. The percentages of AFB1 binding in these biological samples are naturally much below the values obtained from in vitro experiments, but nevertheless positive effects of probiotic administration were obvious in rats and intestinal cells.
Nevertheless, this series of studies has opened a range of questions, which need to be addressed in future work. The administration of aflatoxin and probiotic bacteria in normal food matrix has to be taken into account, since we do not know whether aflatoxin in naturally contaminated crops is accessible for probiotic binding. These bioaccessibility studies could be conducted using the Caco-2 TC-7 cell model. Furthermore, an animal feeding trial needs to be conducted with levels of AFB₁ naturally present in food or feed, to assess effects of probiotics on levels of biomarkers of exposure rather than toxic endpoints, as were used in the present study. This would allow a better prediction of changes to be expected in a human population naturally exposed to dietary aflatoxins. The ultimate goal of this work is to reduce human exposure to aflatoxin and to other harmful food components using probiotic bacteria. This might, in combination with other pre- and post harvest intervention into AFB₁ contamination of food, help to prevent human exposure to aflatoxins and make food more safe.
References


Appendix: Original Publications I-IV
Kuopio University Publications D. Medical Sciences


D 393. Tuhkanen, Hanna. DNA copy number changes in the stromal and epithelial cells of ovarian and breast tumours. 2006. 112 p. Acad. Diss.


