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binding ; bioavailability ; detoxification ; fumonisins ; lactic acid bacteria ; peptidoglycan ; probiotics

ABSTRACT
Aims: The ability of lactic acid bacteria (LAB) to bind fumonisins B_1 and B_2 (FB_1, FB_2) in fermented foods and feeds and in the gastrointestinal tract could contribute to decrease their bioavailability and toxic effects on farm animals and humans. The aim of this work was to identify the bacterial cell wall component(s) and the functional group(s) of FB involved in the LAB–FB interaction.

Methods and Results: The effect of physicochemical, enzymatic and genetic treatments of bacteria and the removal/inactivation of the functional groups of FB on toxin binding were evaluated. Treatments affecting the bacterial wall polysaccharides, lipids and proteins increased binding, while those degrading peptidoglycan (PG) partially decreased it. In addition, purified PG from Gram-positive bacteria bound FB in a manner analogue to that of intact LAB. For FB, tricarballylic acid (TCA) chains play a significant role in binding as hydrolysed FB had less affinity for LAB.
Conclusions: Peptidoglycan and TCA are important components of LAB and FB, respectively, involved in the binding interaction.

Significance and Impact of the Study: Lactic acid bacteria binding efficiency seems related to the peptide moiety structure of the PG. This information can be used to select probiotics with increased FB binding efficiency.

Introduction

Fumonisins, a structurally related mycotoxin group produced by *Fusarium verticillioides* and *Fusarium proliferatum*, are common contaminants of corn and corn-based products worldwide (Shephard *et al.* 1996). There are several identified fumonisins, but fumonisin B_1 (FB_1) and B_2 (FB_2) are the most important and constitute up to 70% of the fumonisins found in naturally contaminated foods and feeds. FB_1 is the diester of propane-1,2,3-tricarboxylic acid (tricarballylic acid, TCA) and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane, in which the C_{14} and C_{15} hydroxyl groups are esterified with the terminal carboxyl group of TCA. FB_2 is the C_{10} deoxy analogue of FB_1, in which the corresponding stereogenic units on the icosane backbone have the same configurations (Fig. 1).

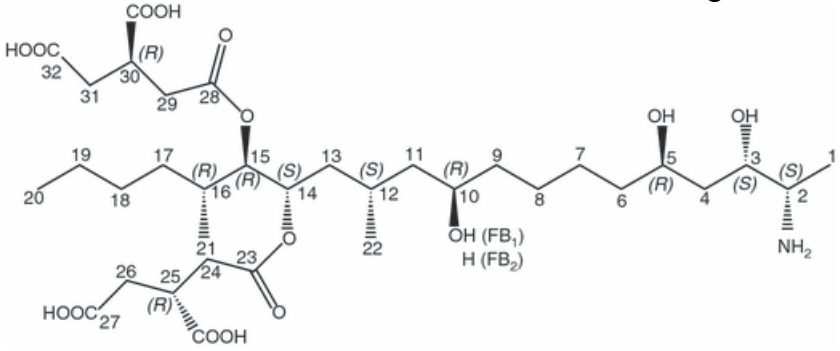

Figure 1 Absolute configuration of fumonisin B_1 (FB_1) and B_2 (FB_2)

Fumonisins B_1 and B_2 are phytotoxic to corn (Lamprecht *et al.* 1994), cytotoxic to various mammalian cell lines (Abbas *et al.* 1993) and FB_1 is a carcinogen in rat liver and kidney (IARC 2002). The occurrence of these analogues in home-grown corn has been associated with an increased risk of esophageal cancer in humans (Shephard *et al.* 2000). FB_1 is considered possible carcinogens to human and classified as class 2B (IARC 2002). These mycotoxins are the causal agent of two well described diseases in domestic animals: equine leukoencephalomacia (Riley *et al.* 1997) and porcine pulmonary edema syndrome (Harrison *et al.* 1990). In addition, they have also been associated with nephrotoxic, hepatotoxic and immunosupressing effects in various animal species (Morgavi and Riley 2007). The mechanism of action appears to involve mainly disruption of sphingolipid biosynthesis by the inhibition of the enzyme sphingosine *N*-acetyltransferase (ceramide synthase) (reviewed by Voss *et al.* 2007). FB are more toxic than their hydrolysed or *N*-acetylated derivatives (Gelderblom *et al.* 1993). The free amino group appears to play a specific role in the biological activity of fumonisins.

Binding of FB by lactic acid bacteria (LAB) from fermented foods and feeds, and by LAB present in the gastrointestinal tract (GIT) could contribute to decrease the toxin bioavailability. This property could also decrease the exposure of intestinal mucosa to FB. Gut tissues exposed to FB have a diminished immune response and an altered barrier function against colonization by pathogenic *Escherichia coli* (Bouhet *et al.* 2004). Viable and nonviable LAB are able to bind FB in a pH, genus, bacterial density and analogue ($FB_2 > FB_1$) dependent manner *in vitro* (Niderkorn *et al.* 2006). FB binding is rapid and particularly effective in acidic conditions, forming a stable complex in the range of pH present in the GIT. This activity is probably present in a variety of fermented foods and feeds (Mokoena *et al.* 2005; Niderkorn *et al.* 2007) and might also operate in the stomach. Binding of other major mycotoxins: aflatoxin B_1 (Haskard *et al.* 2000), zearalenone (El-Nezami *et al.* 2002a) and certain trichothecenes (El-Nezami *et al.* 2002b) by some probiotic LAB has also been shown *in vitro*. In the absence of a simple detoxification method for foods and feeds contaminated by FB, the use of selected strains of LAB appears as a promising approach to reduce their toxicological effects. However, an understanding of the binding mechanism is required to allow the optimization and safe dietary application of this technology. The aim of this work was to identify the component of the bacterial cell wall and the chemical structure of FB involved in the mechanism of binding.

Materials and methods

Bacteria and bacteria-derived materials

Strains *Lactobacillus paraplantarum* CNRZ 1885 (CNRS, FRE2326 Strasbourg, France) and *Streptococcus thermophilus* RAR1 (LAB collection of the Research Unit for Food Process Engineering and Microbiology, INRA, Thivernal-Grignon, France) were used in most experiments. *Streptococcus thermophilus* CNRZ 1066 and its non-capsular, non-exopolysaccharide (EPS) producing mutant *Strep. thermophilus* JIM 8752 (delta epsE) were obtained from the Microbial Genetics Unit, INRA, Jouy-en-Josas, France. *Lactococcus lactis* subsp. *cremoris* MG1363 and mutants, in which the synthesis of certain cell wall components and adhesion properties are affected, were from the LAB and Opportunistic Pathogens Laboratory, INRA, Jouy-en-Josas, France. Bacterial strains were grown at optimal temperature (30 or 37°C) in De Man, Rogosa, Sharpe broth for lactobacilli and M17 broth (Oxoïd Ltd., Basingstoke, UK), supplemented with 0·5% of glucose for lactococci or 10% of lactose for streptococci. Commercial purified peptidoglycans (PG) from Gram-positive bacteria *Micrococcus luteus* and *Bacillus subtilis* were purchased from Sigma, Steinheim, Germany.

Determination of the bacterial cell wall component involved in binding

To identify the binding site, bacteria were subjected to different physicochemical and enzymatic treatments. Bacteria (*Lact. paraplantarum* CNRZ 1885 and *Strep. thermophilus* RAR1) were prepared in advance and stored at −18°C until use. Optimization tests showed that freezing did not negatively affected the binding ability of these strains (shown in results). For experiments, bacteria were thawed at room temperature, washed twice with 0·01 mol l^{-1} phosphate-buffered saline (PBS), pH 7·4 and treated by one of the following methods: water (25 or 100°C, 15 min), hydrochloric acid (1 mol l^{-1} HCl, 100°C, 15 min), sodium dodecyl sulphate (SDS, 2% w/v, 100°C, 15 min) or trichloracetic acid (10% w/v, 100°C, 15 min). After treatment, suspensions were centrifuged (3000 ***g***, 10 min, 5°C). For enzymatic treatments, washed

bacteria were resuspended in 1 ml lysozyme (Sigma; 45 000 U ml^{-1} in phosphate buffer, pH 6), mutanolysin (Sigma; 5000 U ml^{-1} in phosphate buffer, pH 6), pronase E (Sigma; 1 mg ml^{-1} in 0·01 mol l^{-1} PBS, pH 7·4), lipase (Sigma; 1 mg ml^{-1} in 0·01 mol l^{-1} PBS, pH 7·4) or trypsin (Sigma; 1 mg ml^{-1} in Tris-HCl buffer, pH 8, 10 mmol l^{-1} $CaCl_2$). Suspensions were incubated at 37°C for 2 h with shaking (240 rev min^{-1}) and centrifuged (12 000 ***g***, 10 min, 5°C). All bacterial pellets from both the physicochemical and enzymatic treatments were washed three times with 4 ml of PBS and used for the binding assay. Non-treated controls were added at each experimental run. All experiments were performed in triplicate.

Determination of the functional group of fumonisins involved in binding

To identify which functional group of FB can interact with bacteria, different chemical reactions were applied at different sites of FB derivatives. FB_1 and FB_2, purchased from Sigma and Promec (Tygerberg, South Africa), respectively, were dissolved in an exact volume of acetonitrile–water in a 1 : 1 (v/v) ratio to achieve the desired concentration of stock solutions. Hydrolysed FB_1 (HFB_1) and FB_2 (HFB_2) were obtained according to Pagliuca *et al.* (2005). Total hydrolysis of pure FB_1 and FB_2 was checked by HPLC. The chromatograms showed absence of FB peak and presence of a single peak with retention times corresponding to the expected HFB product (Pagliuca *et al.* 2005). An optimized procedure was also used to determine the effect of the amine group in binding. Free amine of both FB was hidden by reaction with *ortho*-phthalaldehyde (OPA). This option was chosen because the fumonisins of the group A in which the free amine is naturally absent are not commercially available.

***In vitro* binding assay**

Treated and non-treated bacteria (10^9 or 10^{10} CFU ml^{-1} for certain experiments, see footnotes of tables) were tested as previously described (Niderkorn *et al.* 2007). Briefly, bacterial material was suspended in 1 ml of corn infusion adjusted to pH 4 with lactic acid and containing FB_1 and FB_2 (5 μg ml^{-1} each) or their derivative compounds. The corn infusion was prepared by steeping dry whole-plant corn in water and filtering as described by Niderkorn *et al.* (2007). For each experiment, positive controls containing no bacterial material and a negative control containing no toxin were included. Assays and controls were incubated at 25°C for 1 h and centrifuged (3000 ***g***, 10 min, 5°C). Supernatants and bacterial pellets were analysed for FB by reversed-phase HPLC to determine free and bound fractions respectively. Because of the instability of the FB-OPA derivative (Williams *et al.* 2004), assays with the free amine hidden were performed following an exact timing: At $t = 0$, a pure FB solution (800 μg ml^{-1}) and reagent with (or without) OPA were mixed (1 : 1 v/v). At $t = 2$ min, 50 μl of this mixture was mixed to 950 μl of acidified corn infusion containing bacteria (10^{10} CFU ml^{-1}), then incubated for 9·25 min at 25°C. At $t = 12$ min, tubes were centrifuged (4500 ***g***, 3 min, 4°C). At $t = 20$ min, supernatants containing free FB were derivatized with OPA. All samples were injected at $t = 22$ min. In these conditions, preliminary assays have shown that the complex FB-OPA remains sufficiently stable to carry out measurements. For this experiment, pellets were not analysed.

Fumonisins analysis

Supernatants from all samples and pellets extracts were fourfold diluted in acetonitrile-water (1 : 1 v/v), then 40 μl were added to 60 μl 0·1 mol l^{-1} borate buffer at pH 10 and 100 μl of OPA reagent were added. The preparation was mixed and allowed to react for 2 min before injection of 20 μl into the HPLC system. For FB extraction, 1 ml acetonitrile–water (1 : 1 v/v) was added to the bacterial pellets and this mixture was vigorously vortexed, placed in an ultrasonic bath for 6 min, then centrifuged (4500 ***g***, 3 min, 5°C). Analysis of FB and their hydrolysed derivatives were done at room temperature by HPLC, using fluorimetric detection. The HPLC system consisted of a GOLD 126 solvent module (Beckman Coulter, Fullerton, CA, USA), an automatic sampler (Spectra-Physics, San Jose, CA, USA) equipped with a 100-μl loop and a fluorescence detector FL3000 (Spectra-System, San Jose, CA, USA). Separation of FB_1, FB_2, HFB_1 and HFB_2 was performed on a C_{18} reversed-phase column (Prontosil, 150 × 4·6 mm, 3 μm, Bishoff Chromatography) with a gradient elution using acetonitrile (A) and water–methanol (1 : 1 v/v) acidified at pH 3·35 with pure acetic acid (B). The gradient was started at 10% of solvent A, which increased to 60% in 6 min, then maintained at 60% for 7 min, before returned to the initial condition in 1 min. The flow rate was 1 ml min^{-1} and detection was set at 336 nm excitation and 440 nm emission.

The retention times of FB_1, FB_2, HFB_1 and HFB_2 were 9·9, 12·2, 10·2, 13·4 min respectively. The percentage of free (or bound) mycotoxin was calculated as 100× [Peak area of mycotoxin in the supernatant (or pellet extract)/Peak area of mycotoxin in the positive control].

Statistical analysis

Data was subjected to the analysis of variance (ANOVA). A significant difference between means of controls and assays ($P < 0·05$) was determined by Dunett's test using the STATISTICAL ANALYSIS SYSTEM (SAS) software package, ver. 8 (SAS Institute Inc., Cary, NC, USA).

Results

Bacterial cell wall components affecting binding

None of the physicochemical treatments applied to bacteria decreased binding of FB_1 or FB_2. On the contrary, freezing/thawing and thermal treatments of bacteria increased the bound fractions of FB_1 and FB_2 in both tested strains ($P < 0·05$) (Table 1). Among the chemical treatments, trichloracetic acid caused a large increase in bound FB proportion ($P < 0·05$). HCl also produced the same effect although it was only significant on *Streptococcus* cells ($P < 0·05$). For the enzymatic treatments, lysozyme and mutanolysin were the only treatments which caused a partial, but significant decrease of this activity ($P < 0·05$) (Table 1). In contrast, lipase, trypsin and pronase E, an unspecific protease from *Streptomyces griseus*, had no effect on binding ($P > 0·05$) (data not shown).

Table 1 Effect of freezing, chemical and enzymatic treatments of bacteria on binding of fumonisin B_1 and B_2 by *Streptococcus thermophilus* RAR1 and *Lactobacillus paraplantarum* CNRZ 1885*

	FB_1						**FB_2**					
	***Strep. thermophilus* RAR1**			***Lact. paraplantarum* CNRZ 1885**			***Strep. thermophilus* RAR1**			***Lact. paraplantarum* CNRZ 1885**		
Treatment	**Free (%)‡**	**Bound (%)§**	**Recov. (%)**	**Free (%)‡**	**Bound (%)§**	**Recov. (%)**	**Free (%)‡**	**Bound (%)§**	**Recov. (%)**	**Free (%)‡**	**Bound (%)§**	**Recov. (%)**
Freezing												
Fresh bacteria (control)	95 ± 3	3 ± 0	98	95 ± 5	2 ± 0	97	64 ± 6	41 ± 3	105	71 ± 0	27 ± 2	98
Thawed bacteria	93 ± 2	14 ± 1 ↑	107	93 ± 1	8 ± 0 ↑	101	41 ± 2 ↑	51 ± 1 ↑	92	60 ± 4 ↑	42 ± 0 ↑	102
Physicochemical treatments†												
Water, room temperature, 15 min (control)	78 ± 5	9 ± 0	87	79 ± 2	9 ± 0	88	30 ± 4	45 ± 1	75	36 ± 5	45 ± 1	81
HCl, 1 mol l^{-1}, 100°C, 15 min	67 ± 1	24 ± 1 ↑	91	87 ± 3	7 ± 2	94	19 ± 2	65 ± 2 ↑	84	35 ± 7	51 ± 7	86
Trichloracetic acid, 10% (w/v), 100°C, 15 min	54 ± 1 ↑	37 ± 1 ↑	91	69 ± 3 ↑	19 ± 4	88	9 ± 2 ↑	76 ± 2 ↑	85	20 ± 3	65 ± 8 ↑	85
Enzymatic treatments†												
Without treatment (control)	91 ± 4	14 ± 1	105	93 ± 3	7 ± 1	100	38 ± 1	53 ± 2	91	65 ± 5	43 ± 2	108
Lysozyme, 45 000 U ml^{-1}	89 ± 3	9 ± 0 ↓	98	93 ± 4	7 ± 0	100	42 ± 11	41 ± 1 ↓	83	75 ± 3 ↓	34 ± 1 ↓	109
Mutanolysin, 5000 U ml^{-1}	92 ± 4	10 ± 1 ↓	102	99 ± 5	5 ± 1 ↓	104	51 ± 7 ↓	42 ± 2 ↓	93	77 ± 7 ↓	27 ± 6 ↓	104

Data shown are means ± SD of triplicates.

↓ and ↑ within the same column, indicate that treatment decreased or increased binding ($P < 0·05$) compared with corresponding control.

*For all experiments, treated or not treated bacteria (10^9 CFU ml^{-1}) were incubated in acidified corn infusion containing FB_1 and FB_2 (5 μg ml^{-1} each) for 1 h at 25°C.

†Experiments were performed with thawed bacteria. Enzymatic treatments were done at 37°C for 2 h.

‡Free fraction of fumonisin remaining in supernatant (*vs* control without bacteria).

§Bound fraction of fumonisin remaining in bacterial pellet (*vs* bacterial pellet spiked with FB_1 and FB_2, 5 μg each).

Table 2 Effect of mutations affecting lipoteichoic acids and peptidoglycan biosynthesis in *Lactococcus lactis* subsp. *cremoris* on binding of fumonisin B_1 and B_2*

Genotype	Protein, function affected or phenotype	FB_1			FB_2		
		Free (%)	Bound (%)	Recov. (%)	Free (%)	Bound (%)	Recov. (%)
Derivatives from *L. lactis* subsp. *cremoris* MG1363							
MG1363	Wild type	92 ± 6	4 ± 1	96	25 ± 2	65 ± 3	90
dltD	LTA synthesis	88 ± 1 ↑	11 ± 1 ↑	99	30 ± 0	69 ± 4	99
pbp 2A−	PBP 2A (PG transpeptidase)	94 ± 1	4 ± 1	98	48 ± 8 ↓	47 ± 1 ↓	95
pbp 2B−	PBP 2B (PG transpeptidase)	95 ± 5	5 ± 0	100	52 ± 6 ↓	45 ± 3 ↓	97

Data shown are means ± SD of triplicates.

↓ and ↑ within the same column, indicate that treatment decreased or increased binding ($P < 0{\cdot}05$) compared with the corresponding control.

PG, peptidoglycan; LTA, lipoteichoic acids; PBP, Penicillin binding protein.

*Bacteria (10^{10} CFU ml^{-1}) were incubated in corn infusion containing FB_1 and FB_2 (5 μg ml^{-1} each) for 1 h at 25°C.

Role of peptidoglycan

We observed decrease of FB_2 binding with mutants of *L. lactis* that had an altered PG structure because of perturbed transpeptidase functions (*pbp* 2A− and 2B−) (Shohayeb and Chopra 1987) ($P < 0{\cdot}05$) (Table 2). However, mutants *acmA* and *ponA* from *L. lactis*, in which the immobilization property (phenomenon of adhesion, chain and biofilm formation) was modified (Mercier *et al.* 2002), had no effect on binding as compared with wild type *L. lactis* ($P > 0{\cdot}05$) (data not shown). To confirm the role of PG component, purified PG from Gram-positive bacteria *M. luteus* and *B. subtilis* at different concentrations (0, 0·1, 0·5, 1 and 1·5 mg ml^{-1}) were tested in a similar way. Results showed that these polymers can bind FB in an analogue dependent manner ($FB_2 > FB_1$) (Fig. 2). Significant bound fractions were observed even with the lowest concentration tested (0·1 mg PG ml^{-1}). However, the binding efficiency varied between the two purified PG tested (*B. subtilis* > *M. luteus*).

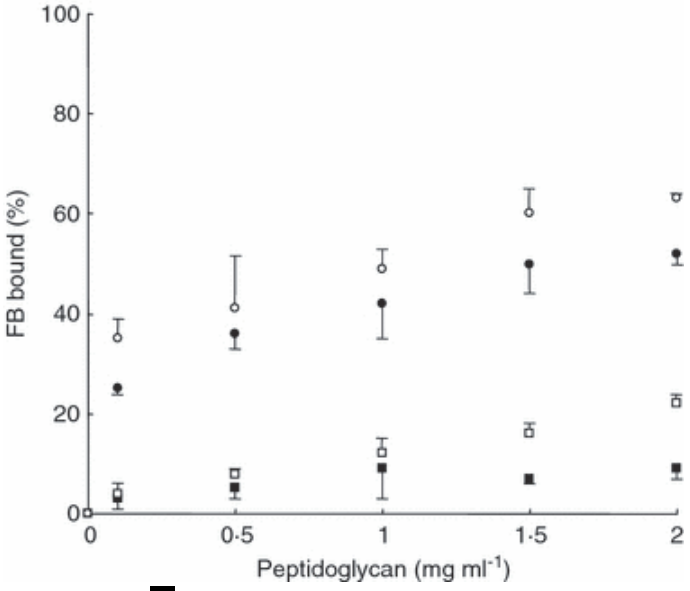

Figure 2 Fractions of fumonisin B_1 (□, ■) and B_2 (○, •) bound to purified peptidoglycans from *Bacillus subtilis* (open symbols) and *Micrococcus luteus* (closed symbols). Data shown are the mean and standard deviations (error bars) of triplicates.

Fumonisins structural component affecting binding

To identify the role of the main functional groups of FB in the formation of the mycotoxin–cell wall complex, the free amine and TCA arms were alternatively hidden or removed. When the free-amine group was hidden by derivatization with OPA, the proportions of FB_1 and FB_2 bound by *Lact. paraplantarum* CNRZ 1885 and *Strep. thermophilus* RAR1 were higher than those observed with unmodified toxins (Table 3). This effect was more pronounced for FB_1 ($P < 0{\cdot}05$) than FB_2 ($P > 0{\cdot}05$). Inversely, the binding rates of HFB_1 and HFB_2 for both strains was lower than those of FB_1 and FB_2 respectively ($P < 0{\cdot}05$). Whatever the applied treatment, binding appears to be greater for FB_2 comparing

with FB_1. To explain the different binding behaviour between FB_1 and FB_2, we investigated their three-dimensional structure by molecular modelling in conditions simulating those of the binding tests. For that, conformations were carried out in aqueous conditions applying ionized states of carboxyl and amine groups of FB in acidic conditions to generate the most stable conformer using MACROMODEL 8.0 (Shroedinger Inc, Portland, OR, USA). Results showed that a hydrogen bond in FB_1 structure is formed between the hydrogen of the hydroxyl group at C_{10} and the oxygen of the carbonyl group of the TCA at C_{15} (Fig. 3).

Table 3 Effect of hydrolysis of fumonisin's tricarballylic acid chains and free amine group inactivation on binding of fumonisin B_1 and B_2 by *Lactobacillus paraplantarum* and *Streptococcus thermophilus**

Toxin	***Lact. paraplantarum* CNRZ *1885***			***Strep. thermophilus* RAR1**		
	Free (%)	**Bound (%)**	**Recov. (%)**	**Free (%)**	**Bound (%)**	**Recov. (%)**
FB_1	73 ± 7	27 ± 2	100	59 ± 1	40 ± 1	99
HFB_1	80 ± 6	14 ± 0 ↓	94	85 ± 10 ↓	22 ± 1 ↓	107
FB_2	19 ± 1	74 ± 1	93	27 ± 1	71 ± 3	98
HFB_2	43 ± 4 ↓	46 ± 11 ↓	89	48 ± 4 ↓	45 ± 3 ↓	93
FB_1†	89 ± 1	ND‡		81 ± 1	ND	
FB_1-OPA	68 ± 2 ↑	ND		66 ± 8 ↑	ND	
FB_2†	56 ± 4	ND		31 ± 4	ND	
FB_2-OPA	48 ± 0	ND		26 ± 0	ND	

Data shown are means ± SD of triplicates.
↓ and ↑ within the same column, indicate that treatment decreased or increased binding ($P < 0{\cdot}05$) compared with the corresponding control.
*Bacterial density = 10^{10} CFU ml^{-1}.
†Treated in the same way as FB-OPA derivatives (see M&M for details).
‡Not determined.

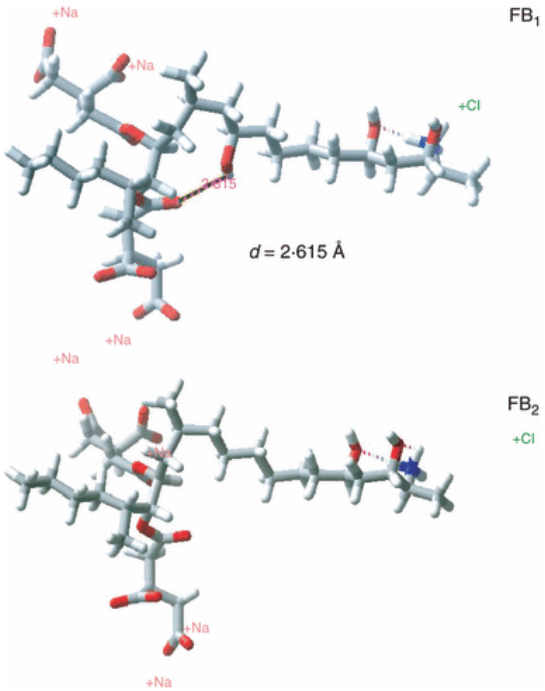

Figure 3 Molecular conformations of FB_1 and FB_2 in aqueous solution. Conformational analysis of molecules in water solution was performed using Monte-Carlo Multiple Method (Chang *et al.* 1989) with AMBER force field (Weiner *et al.* 1984; Cornell *et al.* 1995) and GB/SA solvation model (Still *et al.* 1990) of MACROMODEL 8.0 programme (Shroedinger Inc, Portland, OR, USA). To take account of pKas of fumonisins, conformations were carried out applying ionized states

of carboxyl and amine groups. In this case, four Na^+ and one Cl^- were added in the solution to maintain neutral charge of the molecular system.

Discussion

The ability of LAB to bind fumonisins might contribute to decrease the bioavailability and toxic effects of FB_1 and FB_2 in human and farm animals. The binding activity of LAB could be integrated in the criteria of selection of probiotics and starters used for the acidification of fermented corn meals and corn silage. However, the mechanism of binding is unknown and the LAB–FB interaction needs to be better understood to optimize the selection of strains. In this paper, we provided some insight into the interaction between FB and LAB that can explain different binding behaviour of FB_1 and FB_2.

Determination of the bacterial cell wall binding site

The cell wall of LAB has the typical Gram-positive structure made of a thick, multilayered PG sacculus in which proteins, teichoic acid (TA) and LTA and polysaccharides are associated (Delcour *et al.* 1999). We previously reported that all genera of LAB are capable to bind FB_1 and FB_2 (Niderkorn *et al.* 2007) suggesting that the binding site is a component largely conserved in the cell wall of these bacteria. This component is synthesized early in the bacterial growth cycle since binding was observed in the latency phase. Binding was observed throughout the growth cycle with a maximum at the end of the exponential phase (data not shown).

The increase in binding observed with heat- and acid-treated bacteria (Table 1) was also reported for other mycotoxins such as aflatoxin B_1 (El-Nezami *et al.* 1998) and zearalenone (El-Nezami *et al.* 2002a). It is known that these treatments degrade the surface of the cell wall. The trichloracetic acid treatment to extract PG-associated cell wall polymers from Gram-positive bacteria is well established (Heckels and Virji 1988). Polysaccharides and TA are known targets of HCl and trichloracetic acid treatments (Quiberoni *et al.* 2000). Our results suggest that binding takes place in the subsurface of the cell wall in sites exposed by the heat or acid treatments.

The results obtained with mutant strains are in agreement with the physicochemical and enzymatic treatments of bacteria. Taken together, results indicate that the binding site of FB are not surface polysaccharides, lipids or proteins, but may be rather the PG or compounds tightly associated to it, as it was suggested for the binding of aflatoxin B_1 (Lahtinen *et al.* 2004). It is worth mentioning that LTA are the main components responsible for the hydrophobicity of the cell wall and thus, for the adhesion properties of bacteria (Dahlback *et al.* 1981). However, these adhesion properties appear not to have any function in FB binding. Similarly, the mechanism of immobilization of bacteria, characterized by natural PG modifications consisting of little breaks in the PG structure (Ibrahim *et al.* 2004), seems not to be associated with FB binding.

Binding by commercially available purified PG from two strains of Gram-positive bacteria (Fig. 2) was consistent with our results obtained with intact LAB, thus, supporting the hypothesis that PG is likely the binding site of FB. The PG backbone is a conserved structure composed of linear glycan chains alternating *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc) in a β (1→4) linkage. These chains are crosslinked by means of short peptides. The specific amino acid sequence of peptide bridges and consequently, the molecular structure of PG vary with the bacterial species (Schleifer and Kandler 1972). As the bound fraction of FB varied between the PG tested, but also among genera of LAB (Niderkorn *et al.* 2007), it seems that the amino acid sequence play an important role in the efficiency of the mechanism. The PG structure vary mainly in the amino acid in position 3 (AA_3) of the peptide bridge and in the cross-linking amino acids. *B. subtilis* and *M. luteus* differ in both the AA_3 and cross-linking amino acids (Schleifer and Kandler 1972). This difference could explain their dissimilar efficiency in binding FB. The higher binding efficiency of the *Streptococcus* genus compared with the *Lactobacillus* genus could be because of the amino acid sequence of the cross-bridge that is two to three molecules of L-Ala in the former and D-Asp in the latter (Schleifer and Kandler 1972; Bouhss *et al.* 2002).

Relationship between fumonisins structure and binding

The higher binding rate of the FB-OPA derivative compared with unmodified FB suggest that the free-amine group possessing nucleophilic properties is not involved in FB_1 and FB_2 interaction with bacteria. In addition, in acidic conditions, the ionized state of this function could even decrease binding, in particular for FB_1. Controls containing reagents other than OPA, *e.g.* mercaptoethanol, used in the

derivatization reaction were done to exclude possible interferences on binding. However, the exact function that OPA may have on the FB derivative and/or on the bacterial cell wall that could modify the interaction was unknown. The use of natural fumonisin derivatives such as *N*-acetylated FB_1 might be a better alternative to test the exact role of the free-amino group. Notwithstanding the reservations associated to the derivatization methodology, masking the free-amino group increased rather than decreased binding suggesting that this chemical function has not a positive effect on FB-bacteria interaction. Inversely, the lower binding rate of hydrolysed FB compared with the intact FB indicates that one or both TCA arms play a positive role in the mechanism.

In spite of their similar structure, FB_2 was in all experiments more bound than FB_1 (Tables 1 and 2 and Fig. 2). The same tendency between FB was recorded for OPA-treated or hydrolysed derivatives (FB_2-OPA > FB_1-OPA, HFB_2 > HFB_1) (Table 3). These results are in agreement with those reported for other bacteria and other experimental conditions (Niderkorn *et al.* 2006, 2007). The only structural variation between FB_1 and FB_2 consists in an additional hydroxyl group in C_{10} for FB_1 (Fig. 1). Thus, it is reasonable to postulate that this hydroxyl group plays directly or indirectly a negative role in binding. The spatial conformation induced by the hydrogen bond of FB_1 makes the molecule more coiled and apparently less favourable to binding by the bacterial cell wall. This conformation could disturb the interactions with the PG. These molecular conformations were conserved through pH variation as the addition of charges on functional groups of FB_1 and FB_2 did not affect the results of modelling. However, as HFB_2 was more bound than HFB_1, it seems that the hydroxyl group in C_{10} continues to be unfavourable to binding after TCA removal.

The objective of this work was to improve our understanding of the LAB–FB binding interaction. However, further work is needed before this methodology can be used to treat contaminated feeds. It is important to note that, differently from normal probiotic strains, LAB used to bind FB should have a low capacity to adhere to intestinal mucus and enterocytes to reduce the risk of toxin release in the GIT. The efficiency of bacterial strains to modulate intestinal toxin absorption and toxicity was already demonstrated *in vivo* for aflatoxin B1 in both rats (Gratz *et al.* 2006) and humans (El-Nezami *et al.* 2006).

Conclusions

In this work, we demonstrated that PG of LAB and more generally PG of Gram-positive bacteria, are the most likely site of FB binding. This result helps to explain the widespread binding of fumonisins by LAB. Existing differences in binding capacity of different bacterial species can be rationally explained by the variation in PG structure. This observation should allow to select efficient strains in terms of FB binding, as fermentation starters and/or probiotic mixtures on the base of their PG-type. We also showed that at least one TCA arm of FB play an important role in their binding to bacteria. As it was reported that TCA arms also play a favourable role in the intestinal absorption of FB_1 (Dantzer *et al.* 1999; De Angelis *et al.* 2005), binding of FB_1 and FB_2 could decrease even more their absorption and their toxic effects on the intestinal mucosal cells. However, further quantitative *in vitro* and *in vivo* studies are needed to evaluate the real impact of LAB binding activity on the bioavailability of FB in higher organisms.

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