RESEARCH ARTICLE

Characterization of MabA, a modulator of *Lactobacillus rhamnosus* GG adhesion and biofilm formation

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Abstract

The probiotic *Lactobacillus rhamnosus* GG, first isolated from healthy human gut microbiota, has been reported to adhere very well to components of the intestinal mucosa, thereby enabling transient colonization of the gastrointestinal tract (GIT). In a search for the genes responsible for the good adherence capacity of this strain, a genomic region encoding a protein with homology to putative adhesion proteins (LGG_01865) and its putative regulator (LGG_01866) was identified. The sequence of the *L. rhamnosus* GG *LGG_01865* encodes a polypeptide of 2419 amino acid residues containing 26 repetitive DUF1542 domains and a C-terminal LPxTG cell wall-anchoring motif. Phenotypic analyses of a dedicated *LGG_01865* knockout mutant revealed a reduced biofilm formation capacity on abiotic surfaces and decreased adhesion to intestinal epithelial cells and tissues of the murine GIT. This suggests a modulating role for LGG_01865 in *L. rhamnosus* GG–host interactions. Therefore, we propose a new name for LGG_01865, i.e. MabA, modulator of adhesion and biofilm. Expression analysis indicated that LGG_01866 plays a conditional role in the regulation of *LGG_01865* expression, i.e. when cells are grown under conditions of sugar starvation.

Introduction

The human intestinal microbiota establishes a complex symbiotic interaction with epithelial and immune cells of the gastrointestinal tract (GIT) (Hooper, 2009). In this interaction, the microbiota is essential in providing nourishment, forming a first line of defense against invasion by pathogenic organisms, regulating epithelial development and immune responses. In turn, the host provides stable conditions of temperature, pH, osmolarity and food supply for the microbiota (Leser & Molbak, 2009). Part of the beneficial actions of the GIT microbiota are mediated by their capacity to grow into microcolonies and biofilms (Sonnenburg *et al.*, 2004). Thick-structured bacterial biofilms are generally not observed in the gut of healthy individuals. Rather, microcolonies appear to be the predominant colonization form in these niches (Macfarlane *et al.*, 2004).

A probiotic bacterium is defined as 'a live microorganism that, when administered or ingested in adequate amounts, confers a health benefit on the host' (FAO/WHO, 2001). The origin of probiotic bacteria for human consumption is usually the human gut. In this respect, human consumption of probiotic bacteria aiming to improve or restore the optimal functioning of the microbiota is steadily increasing. An essential characteristic of probiotic bacteria is their capacity to adhere to gastrointestinal surfaces, in this way promoting the transient colonization of the host, pathogen exclusion and interaction with host cells for the enhancement of the epithelial barrier or immune modulation (Servin, 2004). However, the adherence behavior of probiotic bacteria inside the human gut is poorly documented with microscopic studies due to practical issues of obtaining biopsy specimens. In addition, more mechanistic studies are needed on the adhesins used by probiotic bacteria to promote close contact with gastrointestinal surfaces (Lebeer *et al.*, 2008b), including mutational analyses (e.g. Buck *et al.*, 2005).

One of the clinically best-studied probiotic organisms is *Lactobacillus rhamnosus* GG (ATCC 53103), which was

isolated from a healthy human gut microbiota (Doron *et al.*, 2005). *Lactobacillus rhamnosus* GG has been reported to adhere very well to epithelial cells (Tuomola & Salminen, 1998) and immobilized human mucus (Tuomola *et al.*, 1999). Even more, *L. rhamnosus* GG can mediate biofilm formation on abiotic surfaces with an efficiency that exceeds that of related *Lactobacillus* strains (Lebeer *et al.*, 2007b). However, the cell surface molecules of *L. rhamnosus* GG, which are involved in adhesion and biofilm formation, are largely unknown, with the exception of a key role for *L. rhamnosus* GG's pili in these characteristics (Kankainen *et al.*, 2009; Lebeer *et al.*, unpublished data).

In a search for the genes responsible for the good adherence capacity and biofilm formation of *L. rhamnosus* GG, we identified one putative adhesin (LGG_01865) and its divergently transcribed, putative regulatory protein (LGG_01866). In this study, the role of these proteins in *L. rhamnosus* GG adhesion, biofilm formation, *in vivo* persistence and interaction with epithelial cells was investigated using dedicated knockout mutants. Our results clearly demonstrate the modulating role of LGG_01865 in the adhesion and biofilm-formation capacities of *L. rhamnosus* GG. This study is one of the few reports of a functional characterization of an adhesin of a probiotic strain.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus rhamnosus* GG was routinely grown nonshaking in de Man–Rogosa–Sharpe (MRS) medium (Difco) (de Man *et al.*, 1960) at 37 °C. For some assays, Bacto Lactobacilli AOAC medium (Difco) or modified MRS (in which glucose was replaced by another sugar at 20 g L^{-1}) was used as described before (Lebeer *et al.*, 2007b). For some analyses, MRS medium supplemented with 2.5 g L^{-1} pig mucin type II (Sigma-Aldrich) or bile from bovine and ovine (Sigma-Aldrich) at different concentrations was used. *Escherichia coli* and *Salmonella* Typhimurium SL1344 (Hoiseth & Stocker, 1981) were grown in Luria–Bertani medium with aeration at 37 °C (Sambrook *et al.*, 1989). If required, antibiotics were used at the following concentrations: 10 μg mL^{-1} tetracycline, 100 μg mL^{-1} ampicillin and 5 μg mL^{-1} (for LGG) or 100 μg mL^{-1} (for *E. coli*) erythromycin.

DNA manipulations

Routine molecular biology techniques were performed as described before (Sambrook *et al.*, 1989). The PCR primers used in this study were purchased from Eurogentec (Belgium) and are listed in Table 2. Enzymes for molecular biology were purchased from New England Biolabs and used according to the supplier's instructions. Plasmid DNA preparation from *E. coli* was performed using Qiagen miniprep kits. Chromosomal DNA from *L. rhamnosus* GG was isolated as described previously (De Keersmaecker *et al.*, 2006).

Identification and sequence analysis of the *L. rhamnosus* GG *LGG_01865* and the *LGG_01866* gene

The identification of the gene cluster was carried out before the publication of the *L. rhamnosus* GG genome sequence, in a search for putative adhesins. However, because of clarity reasons, we refer to the identified genes with the numbers used in the first *L. rhamnosus* GG genome paper (Kankainen *et al.*, 2009), i.e. *LGG_01865* (*mabA*, encoding a putative adhesin) and *LGG_01866* (encoding a putative regulator). Based on the DNA sequence from a gene encoding a putative adhesin, exoprotein in *Lactobacillus gasseri* ATCC 33323 (*LGAS_0410*, encoding proteinYP_814253) (Azcarate-Peril *et al.*, 2008), primers Pro-97 and Pro-98 (Table 2) were designed, and a probe was constructed using total DNA from *L. rhamnosus* GG as a template, in order to find an *L. gasseri* homolog in an EMBL3 library (λCMPG5317) (as described in Lebeer *et al.*, 2009). Phage DNA of positive clones was purified using the Lambda DNA Extraction Kit (Qiagen, Maryland), and subsequently digested with SalI, BamHI, KpnI and HindIII. The restriction fragments obtained were subcloned and sequenced using the chain termination dideoxynucleoside triphosphate method (Sambrook *et al.*, 1989) with the BigDye® Terminator V3.1 CycleSequencing Kit, using the ABI 3100-Avant Genetic Analyzer (Applied Biosystems). Databases were screened for similarities using BLAST (Altschul *et al.*, 1997), and alignment of overlapping fragments was performed using the VECTORNTI ADVANCE 10 CONTIGEXPRESS software (Informax, Oxford, UK).

Construction of the *L. rhamnosus* GG *LGG_01865* mutant (CMPG5230) and the *L. rhamnosus* GG *LGG_01866* mutant (CMPG5233)

A fragment of 3886 bp, containing a part of *LGG_01865*, was amplified using the primers Pro-212 and Pro-220 (Table 2) and cloned in pCRII-TOPO, yielding pCMPG5228. Subsequently, the amplified fragment was subcloned as an EcoRI fragment in pFAJ5301 (Lebeer *et al.*, 2007a), yielding pCMPG5229. To inactivate *LGG_01865*, a BsaBI/MluI fragment from pCMPG5229 was replaced by the tetracycline resistance cassette *tet* (*M*) amplified previously from plasmid pMD5057 of *Lactobacillus plantarum* 5057 (Danielsen, 2002) using the primers Pro-221 and Pro-222 (Table 2). The resulting suicide vector, pCMPG5230, was electroporated to *L. rhamnosus* GG (De Keersmaecker *et al.*, 2006), and

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant genotype/description	References or sources
E. coli strains		
DH5α	F$^-$ Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^- m_k^+) *sup*E44λ^- *thi*-1 *gir*A96 *rel*A1	Gibco-BRL
Top10F′	F′ (*lacI*^{q}, Tn^r) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*Lac*Z ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(St^r) *end*A1 *nup*G	Invitrogen
Salmonella Typhimurium SL1344	*xyl hisG rpsL*; virulent; Sm^R	Hoiseth & Stocker (1981)
L. rhamnosus GG strains		
Wild type	Human isolate	ATCC 53103 – Gorbach (1996)
CMPG5230	*LGG_01865* knockout mutant of *L. rhamnosus* GG; Δ*LGG_01865*::*tetR*	This study
CMPG5233	*LGG_01866* knockout mutant of *L. rhamnosus* GG; Δ*LGG_01866*::*tetR*	This study
CMPG 5340	Wild-type *L. rhamnosus* GG derivative by insertion of pCMPG5340 at the tRNA^{Leu} locus; Ery^r Tc^r	Lebeer *et al*. (2008a)
Lamda-phage library		
λCMPG5317	EMBL3/BamHI phage containing *L. rhamnosus* GG genomic DNA fragments partially digested with Sau3AI	Lebeer *et al*. (2009)
Plasmids		
pCMPG5228	pCRII-TOPO vector containing a 3886-bp fragment of the *LGG_01865* gene from *L. rhamnosus* GG and its upstream region amplified with primers Pro-212 and Pro-220	This study
pCMPG5229	pFAJ5301 containing the *LGG_01865*–EcoRI fragment from pCMPG5228	This study
pCMPG5230	Suicide vector to knockout the *L. rhamnosus* GG *LGG_01865* gene through insertion of the *tet*^{R} gene from pMD5057 into the BsaBI/MluI site of pCMPG5229	This study
pCMPG5231	pCRII-TOPO vector containing the *LGG_01866* gene and its up- and downstream regions (3015 bp) amplified with primers Pro-219 & Pro-547	This study
pCMPG5233	Suicide vector to knockout the *L. rhamnosus* GG *LGG_01866* gene through insertion of the *tet*^{R} gene from pMD5057 into the XhoI/MluI site of pCMPG5232	This study
pCMPG5506	pCMPG5515 containing the *LGG_01866-LGG_01865* promoter region (900 bp) in the EcoRI site, *LGG_01866* orientation	This study
pCMPG5509	pCMPG5515 containing the *LGG_01866-LGG_01865* promoter region (900 bp) in the EcoRI site, *LGG_01865* orientation	This study
pCMPG5515	pLAB1301 containing the *gus*A promoterless gene from *Lactobacillus gasseri* amplified with primers Pro-70 and Pro-90 in the EcoRI position	This study
pCRII-TOPO	Cloning vector, ampicillin and kanamycin resistance	Invitrogen
pFAJ5301	pUC18 containing the BsaBI/Ecl136II erythromycin cassette from pGK13 in the BspHI site	Lebeer *et al*. (2007a)
pLAB1301	*E. coli–Lactobacillus* shuttle vector, ampicillin and erythromycin resistance	Josson *et al*. (1989)
pMD5057	Tetracycline resistance plasmid from *Lactobacillus plantarum* 5057	Danielsen (2002)

transformants (i.e. double-crossover event) were selected by checking resistance to 10 μg mL^{-1} of tetracycline and sensitivity to erythromycin. Confirmation of DNA recombination was performed by PCR using the primers Pro-536 and Pro-537 (Table 2), and Southern hybridization using a probe synthesized with the primers Pro-226 and Pro-536 (Table 2) (data not shown). A 946 nt internal part of the gene (Fig. 1) was replaced with the tetracycline cassette. The stability of the mutant was confirmed after $>$ 100 generations when grown under nonselective conditions. The *LGG_01865* mutant was designated CMPG5230 and further analyzed (Table 1).

Based on the *LGG_01865* upstream sequence region, a fragment of 3015 bp containing *LGG_01866* and its

Table 2. Primer sequences used in this study

Name	Sequence (5′–3′)	Target sequence	Purpose
Pro-70	TCGAATTCTACTAGAAAGGAAAATCATC ↱	*gus*A gene from *L. gasseri*	Fusion
Pro-90	GCAATTGTCTAGATAATTGAGCACGATTATTTG ↲	*gus*A gene from *L. gasseri*	Fusion
Pro-97	AGCAACTGTTTCGTCAGTCCTAC ↲	Adhesion exoprotein *L. gasseri*	Search for gene
Pro-98	CCCTCGGACTGAAAAGGCTACTC ↱	Adhesion exoprotein *L. gasseri*	Search for gene
Pro-212	TGGCCTGCCCAGACTGGTGC ↲	*LGG_01865* gene	Construction of mutant
Pro-219	GGAATTCTTGTTAAGACTTGTAATAACCGC ↲	*LGG_01865* gene	Construction of mutant
Pro-220	CGAATTCCATAGCGTTGGCTACTTTCG ↱	*LGG_01866* gene	Construction of mutant
Pro-221	GAATTCGAGATTCCTTTACAAATATGCTCTTAC ↱	Tetracycline resistance gene	Insertion *tet*^{R} cassette
Pro-222	CGAATTCGTTCGGAATAGGTTATACTAGACAAAAG ↲	Tetracycline resistance gene	Insertion *tet*^{R} cassette
Pro-226	CCCGGGCTGCCTGATGTTGGGCATCAATGG ↲	*LGG_01865* gene	Construction of mutant
Pro-227	CCCGGGCCTTCGGGTTCATAGGTTCGAATC ↱	Downstream *LGG_1866* gene	Construction of mutant
Pro-232	GAATTCTGAACCCAAAACAGCAAACAATTAAG ↲	*LGG_01865*	Promoter region
Pro-536	CAACACAAGCAAGGCGCTGA ↱	*LGG_01865* gene	Construction of mutant
Pro-537	GCGTCATTAATTTTAGCAGC ↲	*LGG_01866* gene	Construction of mutant
Pro-547	ATTCAGTCAGATGTTGACCC ↱	*LGG_01867* gene	Construction of mutant
Pro-1500	TGGAAACAGTGCTAATACCG ↲	*Lactobacillus* 16S rRNA gene	qRT-PCR
Pro-1501	GTCCATTGTGGAAGATTCCC ↱	*Lactobacillus* 16S rRNA gene	qRT-PCR
Pro-3052	GGCCAAAGTCAAGGATGCTATT ↲	*LGG_01865* gene	qRT-PCR
Pro-3053	CAGTCTTAGCTTTGGTCGCTTCA ↱	*LGG_01865* gene	qRT-PCR
Pro-3054	TTCGCGATTTGGTAACGATAGAT ↲	*LGG_01866* gene	qRT-PCR
Pro-3055	CGCACTTACGCATTTTCTTGAC ↱	*LGG_01866* gene	qRT-PCR

Restriction sites present in the primer sequences are underlined and correspond to EcoRI ↱ and ↲ correspond to reverse and forward primers, respectively.

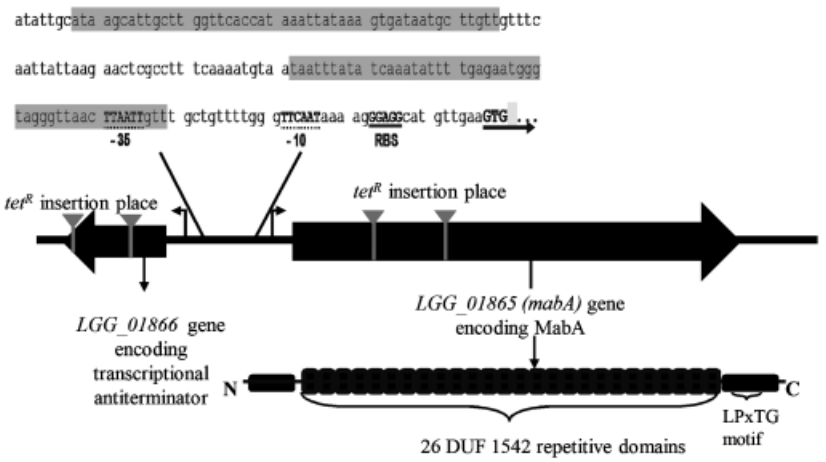

Fig. 1. Genetic organization of *LGG_01865* and *LGG_01866*. ORFs are shown by large arrows. The thin arrows indicate the putative promoter of the *LGG_01865* (*mabA*) and *LGG_01866* genes. The intergenic region is enlarged: a putative Mga-binding site (see text) (highlighted in gray), a putative − 10 and − 35 region (capitalized and dotted-underlined) and a putative ribosome-binding site (RBS) for *LGG_01865* (capitalized and underlined) are suggested. The translational start of *LGG_01865* is capitalized and represented by an arrow. In addition, the tetracycline insertion place, following deletion of part of the coding sequence of the gene, is shown for each gene (see text). Furthermore, the domain structure of LGG_01865, containing 26 repetitive DUF 1542 (domain of unknown function) domains and a C-terminal cell wall-sorting signal with an LPxTG motif, is represented by black squares. The putative transcriptional antiterminator LGG_01866 contains an HTH domain (PF05043).

flanking regions was amplified using the primers Pro-219 and Pro-547 (Table 2), and cloned in pCRII-TOPO, yielding pCMPG5231. Subsequently, the same strategy as that used for the construction of CMPG5230 was followed (i.e. subcloning and gene inactivation). Confirmation of DNA recombination was performed by PCR using the primers Pro-227 and Pro-232 (Table 2). In this mutant, an internal fragment starting with 1140 nt (Fig. 1) was replaced by the tetracycline resistance cassette. The *LGG_01866* mutant was designated CMPG5233 and further analyzed (Table 1).

In vitro adhesion assay to a human intestinal epithelial cell (IEC) line

In vitro adhesion assays using the CaCo-2 cell line were performed as described previously (Lebeer *et al.*, 2009). The adhesion ratio (percentage) was calculated by comparing the number of adherent cells with the cell number of the original bacterial suspension added (10^7 CFU mL^{-1}). Adhesion of *L. rhamnosus* GG wild type, CMPG5230 and CMPG5233 was tested in triplicate in three independent experiments.

In vitro biofilm assay

In vitro biofilm formation assays were performed as described previously (De Keersmaecker *et al.*, 2005; Lebeer *et al.*, 2007a). Data were normalized to the indicated positive control, which was taken as 100% to compare different experiments. Additionally, a sterile medium was used as a negative control. Each strain or condition was tested eightfold. Each experiment was performed at least in triplicate.

Adhesion to extracellular matrix (ECMs) components

Determination of binding of *L. rhamnosus* GG wild type, CMPG5230 and CMPG5233 to immobilized ECM [fibronectin, collagen, fibrinogen, fetuin, lactoferrin and mucin type II – each 100 μg mL^{-1} (Sigma-Aldrich)] was performed as described previously (Beg *et al.*, 2002; Lebeer *et al.*, 2009). Each strain or condition was tested eightfold and each experiment was repeated at least three times.

Inhibition of *Salmonella*-induced inflammatory response in CaCo-2 cells

The experiment was performed as described previously (Coconnier *et al.*, 2000; Nemeth *et al.*, 2006), with minor modifications. Briefly, CaCo-2 cells were preincubated with *L. rhamnosus* GG wild type or mutant strains (10^7 CFU mL^{-1}), followed by a challenge with *S.* Typhimurium SL1344 (10^7 CFU mL^{-1}) for 3 h. Subsequently, RNA was isolated using the High Pure RNA Isolation Kit (Roche) according to the supplier's instructions. The levels of cytokine mRNA induction were measured by quantitative reverse transcriptase (qRT)-PCR and are shown after normalization to the housekeeping gene human peptidyl prolyl isomerase A (hPPIA). *Lactobacillus rhamnosus* GG wild type, CMPG5230 and CMPG5233 were tested in triplicate in three independent experiments.

Survival in simulated gastric juice

Simulated gastric juice was prepared and survival tests were performed as described previously (Lebeer *et al.*, 2008a). The percentages of survival were calculated by comparing the cell numbers before and after addition to simulated gastric juice at 0, 10, 30, 60 and 90 min. Each strain was tested threefold and each experiment was performed at least in triplicate.

Persistence in the murine GIT

The capacity of CMPG5230 and CMPG5233 to persist in the murine GIT was investigated in a competition experiment with *L. rhamnosus* GG wild type (CMPG5340) as described previously (Lebeer *et al.*, 2008a). Groups of five 8-week-old female BALB/c mice for each bacterial strain were obtained from Harlan (Horst, the Netherlands) and housed in conventional filter-top cages. All experiments were performed under the approval of the K.U. Leuven Animal Experimentation Ethics Committee (Project approval number 027/2008).

β-Glucuronidase assay

The *gusA* gene including the putative RBS from *L. gasseri* ADH was amplified using the primers Pro-70 and Pro-90 (Table 2) (Russell & Klaenhammer, 2001) and cloned as an EcoRI–MfeI fragment in the EcoRI site of pLAB1301 (Josson *et al.*, 1989), resulting in plasmid pCMPG5515 (Table 1). The *LGG_01865-LGG_01866* promoter region was amplified using Pro-220 and Pro-232 (Table 2) and cloned in both directions in the EcoRI site of pCMPG5515. pCMPG5506 contains the promoter region in the *LGG_01866* orientation and pCMPG5509 contains the promoter region in the *LGG_01865* orientation (Table 1).

The expression of gene fusions with *gusA* was measured by β-glucuronidase activity as described previously (De Keersmaecker *et al.*, 2006). Bacterial cells, i.e. (1) *L. rhamnosus* GG wild type/pCMPG5506, (2) CMPG5233/pCMPG5506, (3) *L. rhamnosus* GG wild type/pCMPG5509, (4) CMPG5233/pCMPG5509 and (5) *L. rhamnosus* GG wild type/pCMPG5515) (Table 1) in the midexponential phase ($OD_{600\,nm}$ ~0.9–1.0), were used for the analysis. Different environmental conditions were applied, i.e. limitation of O_2 levels, limitation of iron (MRS medium was depleted of iron by adding 18 mM nitrilotriacetic acid trisodium salt), different concentrations of NaCl (0.03% and 0.3%), different temperatures (25 and 37 °C), different concentrations of bile (0.05%, 0.1%, 0.2% and 0.3%), presence of pig mucin type II (2.5 g L^{-1}), lactobacilli AOAC medium and modified MRS medium with or without 20 g L^{-1} sugars (arabinose, maltose, fructose, and galactose). As a control, semi-anaerobic (nonshaking) conditions in MRS medium were used. Each strain and/or condition was tested 24-fold and each experiment was performed in triplicate.

Real-time qRT-PCR

Lactobacillus rhamnosus GG wild type, CMPG5230 and CMPG5233 in the midexponential phase ($OD_{600\,nm}$ ~0.9–1.0) were used for the analysis. Two environmental conditions were applied, i.e. standard MRS medium and MRS medium without 20 g L^{-1} glucose. From each strain, total RNA was isolated using the RNeasy Mini Kit (50) (Qiagen) according to the manufacturer's protocol. Subsequently, cDNA was prepared using the Revert Aid™ H Minus First Strand cDNA Synthesis Kit (Fermentas), according to the protocol provided. The amount of cDNA (three biological repeats) was quantified by real-time qRT-PCR using specific primers for *LGG_01865* (Pro-3052 and Pro-3053) (Table 2) and *LGG_01866* (Pro-3054 and Pro-3055) (Table 2), designed with PRIMER EXPRESS (ABI), and STEPONE (ABI) using PowerSYBR Green PCR Master Mix (ABI), according to the manufacturer's instructions. PCR was performed in a total volume of 20 μL, containing 5 μL cDNA and 15 μL SYBR-GREEN (ABI) combined with 200 nM of the primers. The levels of *LGG_01865* and *LGG_01866* mRNA expression are represented as a ratio after normalization to the 16S rRNA gene (Table 2). All qRT-PCRs were performed in triplicate.

Statistical analysis

To determine significant differences between *L. rhamnosus GG* wild type and the mutants, we used the unequal variance *t*-test. A *P*-value below 0.05 is generally considered as statistically significant.

Results

Identification and annotation of the adhesin and its putative regulator

Based on the gene sequence of *L. gasseri LGAS_0410* (Azcarate-Peril *et al*., 2008), encoding a putative adhesion exoprotein, our hypothesis was that this protein contains domains present in proteins of other lactobacilli and could be used for a search of *L. rhamnosus* GG genes encoding proteins with motifs for adhesion to the intestinal mucosa. Using a reversed genetics approach, EMBL3 clones hybridizing with a PCR probe (see Materials and methods) were isolated and a continuous sequence of 10 kb genomic DNA of *L. rhamnosus* GG was determined. The genetic organization of part of this sequence revealed the presence of two ORFs. Based on homology searches, the first ORF encodes a putative large cell surface protein involved in adhesion to extracellular matrices and the second divergent ORF encodes a putative transcriptional regulator with an Mga helix–turn—helix (HTH) domain (Fig. 1). The recently published *L. rhamnosus* GG genome analyses (Kankainen *et al*., 2009; Morita *et al*., 2009) confirm our earlier dedicated sequence analysis. Kankainen *et al*. (2009) refer to the putative adhesin and the regulator as predicted ORF/conserved ECM-binding protein LGG_01865 and transcriptional antiterminator LGG_01866, respectively. Morita *et al*. (2009) annotated these proteins as LRHM_1797, a putative cell surface protein, and LRHM_1798, a conserved hypothetical protein. For clarity reasons, we will use LGG_01865 and LGG_01866 in the remainder of this text. *LGG_01865* encodes a polypeptide of 2419 amino acid residues containing a C-terminal LPxTG motif recognized by a sortase enzyme, which would be responsible for anchoring of the protein to the microbial surface (Marraffini *et al*., 2006). Domain analysis of LGG_01865 using the Pfam database also revealed the presence of 26 repetitive DUF1542 domains (PF07564) (Fig. 1). The DUF1542 domain represents a series of approximately 75 amino acid residues in length and is found in several cell surface proteins of Gram-positive bacteria (Clarke *et al*., 2002; Schroeder *et al*., 2009). When the sequence of LGG_01865 is compared with sequences in the protein databases, a number of significant matches are found within members of the streptococci, staphylococci and lactobacilli families. The homologous *Lactobacillus* proteins are all functionally uncharacterized. However, within the streptococcal family, homology (~33% identity) was found to Embp (ECM-binding protein) proteins of group A streptococci (GAS) (Manganelli & van de Rijn, 1999). Domain analysis of these proteins reveals the presence of variable numbers of the DUF1542 domain along the sequences, in addition to C-terminal domains. Many homologous proteins have an extra domain (FIVAR), in addition to the DUF1542 domain. This FIVAR domain (PF07554), a sugar-binding domain involved in binding to hyaluronate or fibronectin (Williams *et al*., 2002), is absent in LGG_01865. All identified proteins have a highly repetitive structure and the homology with LGG_01865 is restricted to the repeat region and does not include the N-terminal domain.

LGG_01866 encodes a putative polypeptide of 496 amino acid residues. LGG_01866 shows homology to transcriptional antiterminators (BglG) in other lactobacilli (~50% identity) and to M protein *trans*-acting positive transcriptional regulators (Mga) of *Streptococcus* species (~24% identity), where it regulates the expression of different genes essential for the colonization of host tissues, sugar utilization and positively regulates its own transcription (Hondorp & McIver, 2007). According to Pfam, LGG_01866 contains one HTH domain, the Mga HTH (PF05043) (Fig. 1).

LGG_01865 plays a modulating role in biofilm formation and adhesion by *L. rhamnosus* GG

To investigate the role of the LGG_01865 and LGG_01866 in *L. rhamnosus* GG, isogenic mutants were constructed as

described in Materials and methods and designated as strains CMPG5230 and CMPG5233, respectively (Table 1). The mutants were first phenotypically characterized for their cell morphology by microscopic analyses. No major differences in the morphology were observed between LGG wild type, CMPG5230 and CMPG5233 (data not shown). In addition, no significant effects on the growth characteristics were observed in the mutants compared with the wild type (data not shown). The absence of major morphological and growth defects in these mutants is important for the subsequent phenotypic analysis related to biofilm formation and adhesion, because large surface and adhesin proteins may be involved in the regulation of cell morphology (Popham & Young, 2003).

Lactobacillus rhamnosus GG has been previously shown to exert a high *in vitro* adhesion capacity to IECs (Doron *et al.*, 2005) and a high biofilm formation capacity on polystyrene and glass substrates (Lebeer *et al.*, 2007b). Interestingly, the biofilm formation capacity of both mutants on polystyrene substrates was reduced up to 50% as compared with the wild type in AOAC medium (Fig. 2a). In addition, the LGG_01865 knockout mutant CMPG5230 showed a 40% reduction in adhesion capacity to CaCo-2 cells compared with the LGG wild type (Fig. 2b). In contrast, no significant differences in adhesion to the epithelial cells were observed between the *L. rhamnosus* GG wild type and the *LGG_01866* knockout mutant CMPG5233 (Fig. 2b).

Having established that LGG_01865 is a general adhesin involved in adhesion to CaCo-2 cells and biofilm formation, we subsequently searched for specific ligands of this adhesin, inspired by the ligands of the homologous proteins of LGG_01865 in other species (Manganelli & van de Rijn, 1999; Clarke *et al.*, 2002). Different components of the ECM (fibrinogen, collagen, fibronectin, lactoferrin and fetuin) and mucins were tested in an ELISA-based assay as described in Materials and methods. No significant differences were observed between the wild type and the mutants in the adhesion to immobilized pig mucin type II and tested ECM components (data not shown).

LGG_01865 plays a modulating role in adherence to murine intestine

We subsequently investigated the role of LGG_01865 and LGG_01866 in *in vivo* persistence in the murine GIT by performing a competition experiment with the respective mutants and wild type that were applied in equal numbers to five mice. First, fecal samples were collected as an indication for their survival capacity in the GIT. During the first hours, no significant differences in transit through the GIT were observed between *L. rhamnosus* GG wild type and the two mutants (Fig. 3a and b), i.e. a competitive index of around 1 was calculated for both mutants. Similarly, no differences were observed between *L. rhamnosus* GG wild type and the mutants for survival in simulated gastric juice *in vitro* (data not shown), which is generally a good indicator of the survival capacity of lactobacilli inside the GIT (Lebeer *et al.*, 2008b). Subsequently, the competitive *in vivo* adherence capacity of the mutants was investigated by analyzing the persistence on different tissue parts of the GIT. These *in situ* persistence data showed a difference between both mutants and the wild type. An ~2-fold decrease in the adhesion capacity of the *LGG_01865* isogenic mutant CMPG5230 was observed (Fig. 3c). In contrast, CMPG5233 showed to adhere twofold better than wild-type *L. rhamnosus* GG in the murine GIT (Fig. 3d). These tissue samples were taken 48 h after administration of the bacteria, which corresponds to the last fecal sample taken, where also a small trend toward reduced retrieval of CMPG5230 and increased retrieval of CMPG5233 became apparent.

LGG_01865 does not play a key role in the preventive anti-inflammatory effect of *L. rhamnosus* GG on *Salmonella*-induced inflammation

Salmonella Typhimurium SL1344 induces inflammation in IECs that is characterized by interleukin-8 (IL-8) and tumor-necrosis factor (TNF) production. Several studies have reported an antagonistic activity of probiotic lactobacilli against this induction of proinflammatory responses by *S.* Typhimurium (Nemeth *et al.*, 2006; O'Hara *et al.*, 2006). Having established that preincubation of IECs with the *L. rhamnosus* GG wild type reduces IL-8 and TNF expression (Lebeer *et al.*, unpublished data), we investigated whether LGG_01865 (and LGG_01866) is involved in this effect. No statistically significant differences were observed between the wild type and the *LGG_01865* mutant CMPG5230 (Fig. 4). However, the beneficial effect of *L. rhamnosus* GG wild type pretreatment is no longer observed with CMPG5233 (Δ*LGG_01866*) (Fig. 4).

LGG_01866 is a modulator of *LGG_01865* expression

In *L. rhamnosus* GG, LGG_01866 is encoded upstream of *LGG_01865*, in a divergent orientation (Fig. 1). As mentioned above, LGG_01865 and LGG_01866 show homology to Embp and Mga of GAS, respectively. In GAS, Mga is a global transcriptional activator, which positively regulates the expression of genes encoding ECM-binding proteins (Hondorp & McIver, 2007). In addition, *mga* mutants of GAS exhibited a decreased adherence to human skin tissue and ECM components, similar to the adhesin mutants (Luo *et al.*, 2008; Fiedler *et al.*, 2010). A putative Mga-binding DNA region (Ribardo & McIver, 2006) was found to be located upstream of *LGG_01865*, suggesting that

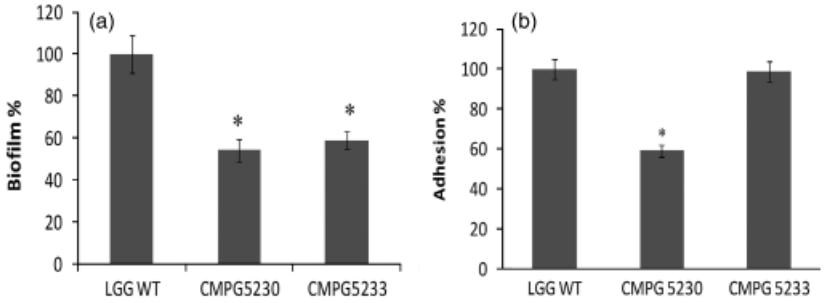

Fig. 2. (a) Biofilm formation of *Lactobacillus rhamnosus* GG wild type, the *LGG_01865* (CMPG5230) and *LGG_01866* mutant (CMPG5233). Biofilm formation is monitored in AOAC medium under anaerobic conditions. The biofilm formation of the *L. rhamnosus* GG wild type was set to 100%. Each experiment was repeated at least three times, and error bars indicate SDs. The dataset comparisons (mutant pairwise to wild type) are considered significant ($P < 0.05$) (indicated with an asterisk in the picture) (b) Adhesion of *L. rhamnosus* GG wild type, *LGG_01865* (CMPG5230) and *LGG_01866* mutant (CMPG5233) mutant to CaCo-2 cells. The adherence percentage is a measure of the ratio of the number of bacteria after adhesion to the initial number of bacteria added to the CaCo-2 cells (*c.* 10^7 CFU mL^{-1}). The adhesion of wild-type *L. rhamnosus* GG was assumed to be 100%. Each experiment was repeated at least three times, and error bars indicate SDs. The dataset comparisons (mutant pairwise to wild type) that are considered significant (see Materials and methods) ($P < 0.05$) are indicated with an asterisk.

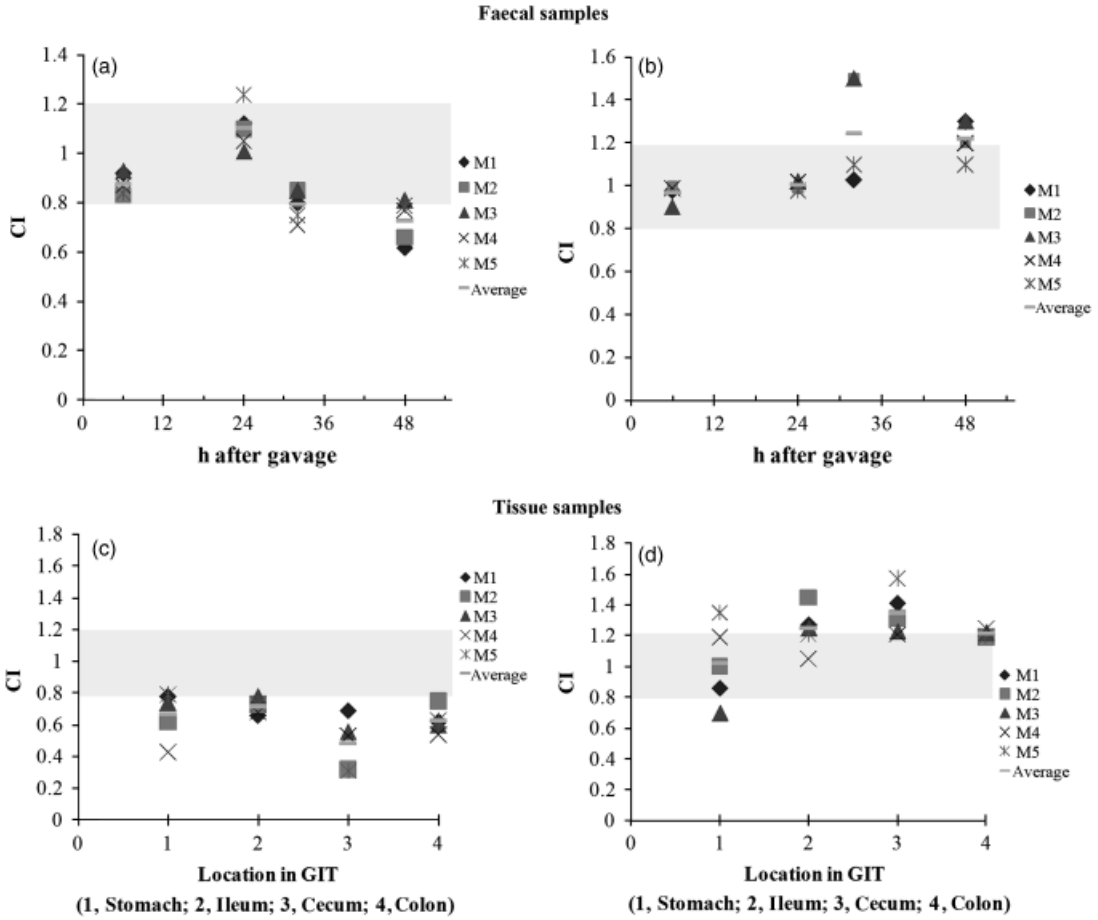

Fig. 3. Characterization of the GIT survival and adhesion capacity of the *LGG_01865* (CMPG5230) and *LGG_01866* mutant (CMPG5233). The competitive index (CI) is the ratio obtained after passage through the GIT compared with the actual ratio of the wild-type control and mutant before gavage and recalculated using a corresponding correction factor. The gray zone shows the normal border for CI (between 0.8 and 1.2), which corresponds to an initial ratio of 1 : 1 of the mutants and the wild-type *Lactobacillus rhamnosus* GG upon administration. The values for individual mice (M1–M5) are shown. (a and b) Comparison of the wild-type control vs. CMPG5230 (a) and CMPG5233 (b) recovery in fecal samples. (c and d) Comparison of wild-type control vs. CMPG5230 (c) and CMPG5233 (d) for adhesion to tissue samples from different locations of the murine GIT.

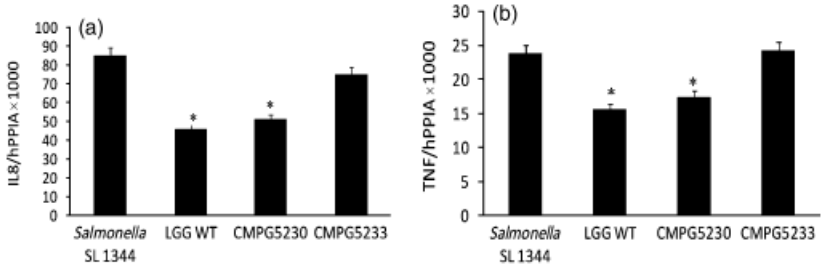

Fig. 4. Anti-inflammatory effect of *Lactobacillus rhamnosus* GG wild type, the *LGG_01865* (CMPG5230) and *LGG_01866* mutant (CMPG5233) against *Salmonella* Typhimurium SL1344. All the cells were administered at 10^7 cells mL^{-1}. The levels of IL-8 and TNF are presented as a ratio after normalization to the housekeeping gene hPPIA. Each experiment was repeated at least three times, and error bars indicate SDs. (a) IL-8; (b) TNF. Significant differences ($P < 0.05$) are indicated in the picture with an asterisk.

LGG_01866 could be an activator of *LGG_01866* expression (Fig. 1). However, comparative mutant analysis of CMPG5230 (Δ*LGG_01865*) and CMPG5233 (Δ*LGG_01866*) described above showed that their phenotypes are different with respect to adhesion to IECs, adherence to murine GIT tissue and immunomodulation. Therefore, we investigated the importance of LGG_01866 for *LGG_01865* expression in *L. rhamnosus* GG. Firstly, two different *gusA* fusions containing the *LGG_01865* and *LGG_01866* promoter regions in their corresponding orientations (i.e. P_{LGG_01865}::*gusA* and P_{LGG_01866}::*gusA*) were constructed. The resulting β-glucuronidase activity was tested under different environmental conditions and in different genetic backgrounds, i.e. LGG wild type, vs. *LGG_01866* mutant (CMPG5233) as described in Materials and methods. P_{LGG_01865}::*gusA* and P_{LGG_01866}::*gusA* are expressed under all the conditions tested, with a small decrease under conditions of stress (i.e. AOAC medium, sugar starvation, presence of bile). Only under conditions of sugar starvation (i.e. modified MRS with sugars that are not fermentable by *L. rhamnosus* GG, or modified MRS without sugar) could a significant reduction in P_{LGG_01865}::*gusA* expression in the *LGG_01866* mutant background (CMPG5233), in comparison with the wild-type background, be observed (Fig. 5a). However, LGG_01866 does not seem to be strictly required for *LGG_01865* expression, which indicates that other factors are involved in regulating its expression. In GAS, Mga has also been shown to autoregulate *mga* expression positively (Ribardo & McIver, 2006). For LGG_01866, we could observe, under some conditions (AOAC medium, presence of bile and mucin), a small decrease in the P_{LGG_01866}::*gusA* in the *LGG_01866* background (CMPG5233), compared with the wild-type background (data not shown). This suggests that *LGG_01866* is positively autoregulated under these conditions. However, under conditions of sugar starvation, LGG_01866 seems to be a negative autoregulator (Fig. 5b).

We also performed real-time qRT-PCR to measure *LGG_01865* and *LGG_01866* expression in the different genetic backgrounds. It needs to be mentioned that this method quantifies cDNA, while the GusA experiment determines enzyme activity. However, both methods provide indications of the expression of the *LGG_01865* and *LGG_01866* genes. Firstly, these qRT-PCR data show that the mutations have no polar effect, as the expression of *LGG_01866* is not affected in the *LGG_01865* mutant (CMPG5230) (Fig. 5d), and the expression of *LGG_01865* is not affected in the *LGG_01866* mutant (CMPG5233) under nonregulating (i.e. no sugar limitation) conditions (Fig. 5c). Secondly, the qRT-PCR results substantiate the results of *gusA* reporter gene fusion experiments, indicating that LGG_01866 is involved in the positive regulation of *LGG_01865* expression under conditions of sugar limitation (Fig. 5c). Indeed, the quantity of *LGG_01865* mRNA is decreased in the *LGG_01866* mutant, compared with the wild type (Fig. 5c). However, *LGG_01865* is still expressed in the *LGG_01866* mutant, demonstrating that other factors, in addition to LGG_01866, are involved in *LGG_01865* regulation, again confirming the results of *gusA* reporter gene fusion experiments.

Discussion

An important characteristic for probiotic bacteria is the ability to adhere to different parts of the intestinal mucosa, i.e. epithelial cells, mucus layer and ECM components (Perea Vélez *et al.*, 2007). Different cell wall molecules may be involved in adhesion to and interaction with the host in a multiple-step process. After an initial step of adhesion, a more stable interaction with the host surface is estimated. Thereafter, bacteria are able to initiate the formation of microcolonies by embedding themselves in an exopolymeric matrix that eventually results in the formation of a thick and structured biofilm. To close this cycle, bacteria can detach from the surface (dispersion) and colonize new niches (Monds & O'Toole, 2009). All these steps can be observed in the gut, but the process is generally limited to

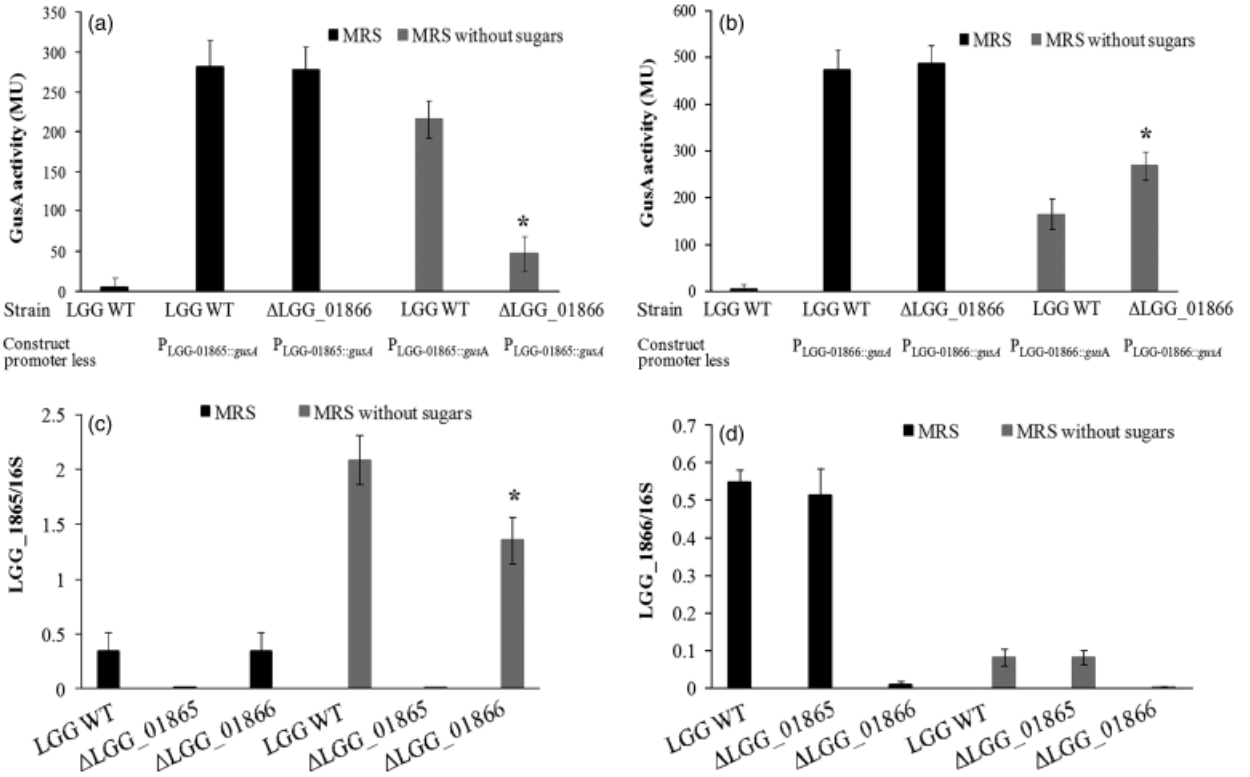

Fig. 5. Expression analyses of *LGG_01865* and *LGG_01866*. β-Glucuronidase activity of the transcriptional fusions P_{LGG_01865}::*gusA* (a) and P_{LGG_01866}::*gusA* (b). β-Glucuronidase activity (GusA) is expressed in Miller units (MU) and under two conditions – MRS medium and MRS without sugars (sugar starvation). Similar results were obtained with modified MRS containing sugars, that are not fermentable by *Lactobacillus rhamnosus* GG (data not shown). (a) P_{LGG_01865}::*gusA* expression in the Δ*LGG_01866* mutant background (CMPG5233), in comparison with the wild-type background; (b) P_{LGG_01866}::*gusA* expression in the Δ*LGG_01866* mutant background (CMPG5233), in comparison with the wild-type background. Each experiment was repeated at least three times, and error bars indicate SDs. Significant differences ($P < 0.05$) are indicated in the picture with an asterisk. The wild type containing the promoterless *gusA* construct (CMPG5515) showed no β-glucuronidase activity (negative control). (c and d) Real-time qRT-PCR expression analysis. (c) Levels of *LGG_01865* mRNA in the Δ*LGG_01866* (CMPG5233) and Δ*LGG_01865* (CMPG5230) mutants, in comparison with the wild type. (d) Levels of *LGG_01866* mRNA in the Δ*LGG_01866* (CMPG5233) and Δ*LGG_01865* (CMPG5230) mutants, in comparison with the wild type. The levels of *LGG_01865* and *LGG_01866*mRNA are presented as a ratio after normalization to the 16S rRNA gene under two different conditions, i.e. MRS medium and MRS without sugars (sugar starvation). Each experiment was repeated at least three times, and error bars indicate SDs.

multispecies microcolony formation in healthy individuals (Macfarlane *et al.*, 2004; Swidsinski *et al.*, 2005).

The first characterized adhesin in *L. rhamnosus* GG is part of the pili (Kankainen *et al.*, 2009). The current report describes the characterization of the *L. rhamnosus* GG large protein LGG_01865, with homology to adhesion proteins, and its putative regulator LGG_01866. This is the second adhesin of *L. rhamnosus* GG that has been functionally characterized through the construction of dedicated knock-out mutants, i.e. CMPG5230 (*LGG_01865* mutant) and CMPG5233 (*LGG_01866* mutant). The *LGG_01865* mutant CMPG5230 showed a *c.* twofold reduced adherence capacity to CaCo-2 cells and *c.* twofold reduced *in vitro* biofilm-formation capacity. The differences in the adhesion between wild-type *L. rhamnosus* GG and CMPG5230 *in vivo* showed that LGG_01865 also plays a modulating role for adhesion to murine GIT tissues, highlighting a role for LGG_01865 as an adhesin. However, in contrast to the *L. rhamnosus* GG pilin mutants (Kankainen *et al.*, 2009; Lebeer *et al.*, unpublished data), CMPG5230 can still adhere to the CaCo-2 cells and form biofilms, suggesting that LGG_01865 acts more like a modulator of adhesion and biofilm formation compared with the pili. We hypothesize that LGG_01865 plays a role in the later steps in the adhesion processes, i.e. in the formation of more stable interactions with biotic and abiotic surfaces after the pili have made the first contact (i.e. 'close-distance contact' hypothesis).

Having established that LGG_01865 has a general modulating function in adhesion to epithelial cells and biofilm formation and based on the homology to other proteins in *Streptococcus* and *Staphylococcus* that mediate adhesion to ECM proteins, we attempted to find a specific ligand for

LGG_01865 using different components of ECM. However, we could not identify a specific ligand among the obvious candidates tested (porcine gastric mucins, fibronectin, collagen, fibrinogen, fetuin and lactoferrin). This is probably related to the absence of the putative ECM-binding FIVAR domain (PF07554) (Williams *et al.*, 2002) in LGG_01865. Finally, we compared the anti-inflammatory capacity of the CMPG5230 mutant with *L. rhamnosus* GG wild-type IECs challenged with *Salmonella*, because pretreatment with *L. rhamnosus* GG wild type inhibits the expression of IL-8 and TNF after challenge of the CaCo-2 cells with *Salmonella* (Coconnier *et al.*, 2000). LGG_01865 is predicted to be surface located and contains repetitive domains. Hence, this makes this protein an ideal candidate to be involved in the probiotic–host interactions and modulate cytokine expression in host cells (Lebeer *et al.*, 2010). However, no differences were observed between the *L. rhamnosus* GG wild type and CMPG5230 were observed, showing that LGG_01865 is not important for this anti-inflammatory effect of *L. rhamnosus* GG.

The role of LGG_01866 in the adhesion process of LGG is more difficult to interpret. LGG_01866 appears to be required for optimal biofilm formation on polystyrene substrates as a 50% decrease in biofilm formation was observed for the *LGG_01866* mutant CMPG5233 in AOAC medium, similar to the decrease observed for CMPG5230. However, in contrast to LGG_01865, LGG_01866 is not required for adhesion to CaCo-2 cells, also highlighting that biofilm formation is a more complex process than merely adhesion to substrates. In addition, the results for the *in vivo* persistence capacity of CMPG5233 in the murine GIT indicate that CMPG5233 adheres better *in vivo* compared with the *L. rhamnosus* GG wild type and CMPG5230. Finally, in contrast to the *LGG_01865* mutant CMPG5230, CMPG5233 was also shown to impact on the anti-inflammatory capacity of LGG in IECs. A possible explanation for these results is that the *LGG_01866* mutation has pleiotropic effects in *L. rhamnosus* GG. LGG_01866 could control the transcription of many other determinants involved in adhesion *in vivo* and cytokine-modulating interactions with IECs. The phenotypes of the CMPG5230 and CMPG5233 are thus not alike, except for biofilm formation, highlighting that LGG_01866 does not merely act in *L. rhamnosus* GG as a positive regulator of *LGG_01865* expression, as was first hypothesized based on the genome location (next to each other and divergently described) and on their homology to Mga and ECM-binding proteins in several pathogens, where the positive regulation of these proteins by Mga has been demonstrated (Terao *et al.*, 2001). Using *gusA* expression analyses and real-time qRT-PCR, we established that LGG_01866 is involved in the positive regulation of *LGG_01865* expression only when bacterial cells are exposed to stress, like sugar starvation and the presence of bile. This could provide to *L. rhamnosus* GG the possibility to adhere better to the GIT mucosa under these conditions. However, our expression studies indicate that LGG_01866 is not the only regulator controlling *LGG_01865* expression under the conditions tested, suggesting that the regulation of LGG_01865 is multifaceted. In GAS, Mga also plays a pleiotropic role (Kreikemeyer *et al.*, 2003; Luo *et al.*, 2008; Fiedler *et al.*, 2010). Further experiments, including genome-wide expression analyses of CMPG5233 under different conditions, will have to shed light on the complex regulatory role of LGG_01866 in *L. rhamnosus* GG.

In conclusion, the current report describes the identification and characterization of LGG_01865 and a conditional regulator of its transcription, LGG_01866. Phenotypical *in vitro* and *in vivo* analyses of the corresponding knockout mutants indicate that LGG_01865 of *L. rhamnosus* GG plays an important modulating role in adhesion to IECs and biofilm formation. Based on our results, we propose a new name for LGG_01865, i.e. MabA (modulator of adhesion and biofilm). Furthermore, analyses of the *LGG_01866* mutant indicate that LGG_01866 could be an important pleiotropic regulator in *L. rhamnosus* GG in phenotypes related to biofilm formation, adhesion *in vivo* and immunomodulation in IECs. Future studies will provide more information on the potential role of LGG_01866 as a transcriptional regulator of other probiotic factors important for health benefits of *L. rhamnosus* GG.

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Authors' contribution

M.P.V., M.I.P. and S.L. contributed equally to this work.

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