World Journal of Dairy & Food Sciences 9 (1): 70-78, 2014
ISSN 1817-308X

DOI: 10.5829/idosi.wjdfs.2014.9.1.81278

Identification of *Lactobacillus rhamnosus* GG Bacteriocin Gene Determinants Expressed *In vivo* in Murine Gut

Akruti Joshi, Senan Suja and Prajapati Jashbhai

Department of Dairy Microbiology,
Sheth MC College of Dairy Science, Anand 388110 Gujarat, India

Abstract: Categorical confirmation of *L. rhamnosus* GG (LGG) bacteriocin production capabilities is not yet established. The main objective of this study was to investigate whether the probiotic strain LGG bacteriocin encoding genes are induced inside the murine GIT using Resolvase based *In vivo* Expression Technology. Bioinformatics led to the finding that LGG contain a lantibiotic belonging to the pediocin like family (LGG_02400) and class II b (LGG_02391-02392). A 200 bp genomic fragment containing the putative promoter of the genes LGG 02385-LGG_02386 encoding for bacteriocin export ABC transporter was amplified and the fragment was cloned at the XhoI-BglII site upstream of the promoterless *cre* gene in plasmid pCMPG5350 resulting in plasmid pCMPG5367. This plasmid was subsequently transformed to the reporter strain CMPG5340-Rif^R, containing the *lox*P- flanked Tc^R marker gene by electroporation. For analysis of the induction of bacteriocin promoters, two mice received a 100µl oral dose 2×10^{10}cfu of these freshly prepared bacterial suspensions by intragastric administration on 2 consecutive days. It was observed that less than 0.5% of the colonies from the 48 h time point were tetracycline sensitive. This is probably related to the fact LGG showed a low rate of cell division in the murine GIT and one cell division is needed for each Cre-mediated excision. It can be concluded that the expression of the promoter is not in a constitutive form. These, results support that bacteriocin may serve as an adaptation factor for the survival and persistence of *L. rhamnosus* GG inside the gastrointestinal tract of mice.

Key words: RIVET · LGG · Bacteriocin · Cre-Lox · Probiotic

INTRODUCTION

Lactic Acid Bacteria (LAB) plays a role in control of large number of disorders ranging from gut restoration to brain functioning. Lactobacilli (a member of LAB) exert health benefits on gut, skin, urinogenital tract and even brain functioning [1].

Lactobacillus rhamnosus strains have been isolated from various ecological habitats; develop distinctive metabolic traits to compete with other bacterial species by producing bacteriocins that prevent growth of other bacterial populations.. *Lactobacillus rhamnosus* GG (ATCC 53103, Valio, Finland) (LGG) is one of the commercially important probiotic strain with the largest number of proven health benefits [2], prevent and relieve certain types of diarrhea [3] and atopic disease [4] and reduce inflammation in some milder states of inflammatory bowel diseases [5]. Silva *et al.* [6] demonstrated a potent inhibitory activity of *L. rhamnosus* GG against a wide range of bacterial species including anaerobic bacteria such as *Clostridium* spp. *Bacteroides* spp. *Bifidobacterium* spp. members of the family *Enterobacteriaceae*, *Pseudomonas* spp. *Staphylococcus* spp. and *Streptococcus* spp. but was not able to inhibit other Lactobacilli.

Molecular information is needed on factors that promote survival and adaptation of LGG in the GIT. Detailed analysis of bacteriocin genes of LGG has not yet been performed. This stimulates the search for the presence, activity and mechanism of bacteriocin production by LGG inside the mammalian host. In this study, based on bioinformatic evidence the role of the

Corresponding Author: Suja Senan, Dairy Microbiology Department Sheth MC College of Dairy Science, Anand Agricultural UniversityAnand - 388 110 India.
Tel: +917567336611.

putative bacteriocin (s) of LGG for survival and persistence in the GIT will be investigated by *in vitro* experiments, as well as *In vivo* conditions in the murine GIT. Recombinase based *In vivo* gene expression technology (RIVET) is a variant of the original *In vivo* expression technology in which a promoter transcriptional event is captured permanently as a conversion of the infecting strain from antibiotic resistant to antibiotic sensitive. Using RIVET we have applied a genome-wide genetic screening approach to investigate whether LGG was able to produce bacteriocin in presence of competing microbes and to check whether bacteriocin encoding genes are expressed during an active murine infection. This is the first time that an RIVET strategy has been applied to *LGG.*

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions: The bacterial strains and plasmids used in the present study are listed in Table 1. All *Lactobacillus* strains were grown at in MRS (Difco) or Lactobacilli AOAC medium (Difco) in non-shaking conditions at 37 °C. *E. coli* strains were grown in Luria-Bertanii broth at 37°C in shaking conditions. When required, antibiotics were added to media in the following concentrations: 10 µg/ml of tetracycline, 100 µg/ml of ampicillin, 5 µg/ml of erythromycin (For LGG). Culture tubes (Diameter, 16 mm) containing 5 ml of prewarmed medium were inoculated with 2% of an overnight culture and incubated at 30°C without shaking in a water bath. Bacterial growth was monitored by estimating viable count as well as by spectrophotometric measurements of the optical density at 600 nm (Model UV-1205; Shimadzu, Kyoto, Japan) at intervals of 0,3,6,9,12,16,18 and 24 hours. Growth curves were plotted using the data derived by optical density versus the colony forming units of the respective cultures. The growth curve of each strain was constructed from the results of six to eight independent experiments.

DNA Manipulation Techniques and Sequence Analysis: Routine molecular biology techniques (Restriction digests, polymerase chain reaction, DNA ligation, transformation to *E. coli,* etc.) were performed according to standard procedures [7]. Bacteriocin genes were characterized by the use of BLAST protein analysis that identified the related sequences present in the other *Lactobacillus* species which are proved for bacteriocin production and BAGEL which is an important bioinformatic tools to find genes responsible for putative bacteriocin production. BAGEL2 is freely accessible at *http://bagel2.molgenrug.nl*. In addition to bacteriocin mining, the website offers ORF prediction tools and a BLAST server that can be used to BLAST search against the bacteriocin database.

Primer Construction: Primers were constructed for the promoter probe constructs through the Vector NTI database software. Vector NTI is a well-balanced desktop application integrated for molecular sequence analysis and biological data management.

Construction of Promoter Probe Constructs Based on the *Cre-lox* System: A 200 bp genomic fragment containing the putative promoter of the genes *LGG 02385-LGG_02386* which encodes for components of a putative bacteriocin export ABC transporter was amplified with primers Pro-02371 (*Bgl*II ends) and Pro-02372 (*Xho*I ends) (Table 2) using genomic DNA of LGG as a template. This fragment was cloned in the *Xho*I-*Bgl*II site upstream of the promoterless *cre* gene in plasmid pCMPG5350, resulting in plasmid pCMPG5367 (Fig. 1). This plasmid was subsequently transformed to the reporter strain CMPG5340-RifR, containing the *loxP*- flanked TcR marker gene by electroporation as described before [8].

Recombination-based *in Vivo* Expression Technology (RIVET) Animal Experiments Procedure: Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and were reviewed and approved by the The *Katholeikke University of Leuven* Institutional Animal Care and Use Committee. Groups of two BALB/C mice 8-10 weeks old were purchased from Harlan (The Netherlands) and housed in conventional filter top cages at the Animalium of University Hospital of the K.U. Leuven. Food and water supply were given ad libitum. For analysis of the induction of bacteriocin promoters, two mice received a 100 µl oral dose 2×10^{10} cfu of these freshly prepared bacterial suspensions by intragastric administration on two consecutive days. After the last administration, individual fecal samples were collected in screw cap tubes. Subsequently, the mice are sacrificed and the content and tissue samples were collected in falcon tubes. After extensive homogenization, the samples were stored at -80°C and the fecal samples were plated by making appropriate dilutions of the suspensions on MRS plates containing 50 mg/ml of rifampicin. After 72 h, full-grown colonies were replica plated onto plates containing 5 mg/ml of chloramphenicol and 10 mg/ml of tetracycline

Table 1: Strains and plasmid used in this study and there relevant characteristics

Strains	Relevant features or sequence	Source
E.coli strains DH5α	F- 80dlacZM15 (lacZYA-argF)U169 deoRrecA1 endA1 hsdR17(r_k-,m_k-) supE44 thi-1 γ-gyrA96 relA1 -	Gibco-BRL
L.rhamnosus GG	Wild type, originally isolated from a faeces sample form a healthy adults, genome sequence is available	ATCC 531303
L.rhamnosusRif resistant CMPG 5340	Rif r derivative of CMPG5340 (CMPG5340 = wild-type LGG derivative by insertion of pCMPG5340 at the $tRNA^{leu}$ locus, Ery^R, $Tc^{R;}$ pCMPG5340 = pEM40 derived integration vector containing the loxP-tetR-loxP marker with the tetR gene from pMD5057)	[29]
L. rhamnosusATCC 9595	Wild type strain	ATCC
L. rhamnosusATCC 7469	Wild type strain	ATCC
L. rhamnosusATCC 10863	Wild type strain	ATCC
L. caseiATCC393	Wild-type strain, neotype strain; ; dairy strain	ATCC
L. caseiATCC334	Wild-type strain, also classified as L. zeae; dairy strain	ATCC
L. paracasei	Ribotyped by VTT; isolated from Columbian yoghurt Alpina baby, Pasco	[32]
L. plantarumWCFS1	Wild type strain; originally isolated from human saliva sample WageningenUniversity	
L. acidophilus	Ribotyped by VTT origin; Yoghurt Finesse (F13) Alpina. Columbia	[32]
L.sake Lb706	sakacinA, producer of bacteriocin	Prof.Michelis
L.sake Lb45	lactocinS producer of bacteriocin	Prof.Michelis
L. johnsoniiVPI11088	Lactacin F, producer of bacteriocin	Prof.Michelis
L.plantarum WCFS1	Wild type strain	NCIMB
L.sakeATTC 15521	Bioassay strain	ATTC
Salmonella typhimurium	Wild type strain	SL1344
Plasmids		
pCMPG5350	pLAB1301 derivative containing the CmRfrom pGIZ850 instead of the EryR marker and the cre gene from pNZ7125; promoter probe vector for R-IVET , E. coli-Lactobacillus shuttle vector; Ap^R,Cm^R	This work
pCMPG10205	pFAJ5301+ Tc resistance cassette (XbaI, Bam HI), construct for making knockout mutants with double homologous recombination. Multiple cloning sites, up- and downstream of Tc^R- cassette	This work
pCMPG5367	Plasmid containing the promoter of the bacteriocin genes LGG_02385-LGG_02386 cloned in the XhoI-BglII site upstream of the promoterlesscregene in plasmid pCMPG5350	This work
pCRII-TOPO	Cloning vector, Ap^R	Invitrogen

Table 2: Primers used in this study and there relevant characteristics

Primers of promoter probe constructs	Sequence 5'-3'	Description of primers
Pro-2371	ATCTCGAGTGATCTGGCCTAG	Forward primer of LGG_02386-LGG_02385 genes
Pro-2372	ATAGATCTTTGGCTAGTTTTCT	Reverse primer of LGG_02386-LGG_02385 genes
Primers for constructing mutants	Sequence 5'-3'	Description of primers
Pro 3223	ATCCCGGGAAGCGAAACGCAGGTCACT	Forward primer 1 of LGG_02385 and LGG_02386 genes
Pro 3224	ATCCCGGGCATCAGCCTCTCCAGAAC	Reverse primer 1 of LGG_02385 and LGG_02386 genes
Pro 3225	ATGCGGCCGCGAAAATGCGATGCCCA	Forward primer 2 of LGG_02385and LGG_ 02386 genes
Pro3226	ATGTCGACCAACGCATCCAATTGTCAG	Reverse primer 2 of LGG_02385 and LGG_02386 genes
Primers for TcR genes	Sequence 5'-3'	Description of primers
Pro-221	GATTCGAGATTCCTTTACAAATATGCTCTTAC	Forward primer tet(M) gene of L. plantarum MD5057
Pro-222	CGAATTCGTTCGGAATAGGTTATACTAGACAAAAG	Reverse primer tet(M) gene of L. plantarum MD5057

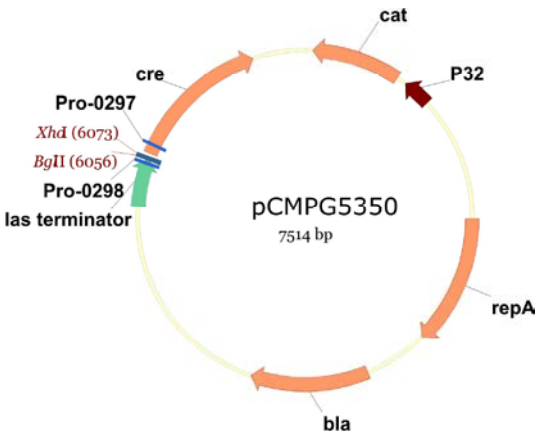

Fig. 1: L. rhamnosus GG-derivative strain, CMPG5340-RifR containing pCMPG5367

Another 24 h later; the plates were compared, leading to the identification of cells displaying a tetracycline-sensitive phenotype. These sensitive colonies were further tested by PCR amplification, using primers Pro- 221 as forward primer and Pro-222 as reverse primers which amplifies the *tet (M)* gene of interest.

RESULTS AND DISCUSSION

Sequence Analysis of the Bacteriocin Genes: De Keersmaecker *et al*. [8] demonstrated that the spent culture supernatant (SCS) of the probiotic *Lactobacillus rhamnosus* GG has been found to exert antibacterial activity against *Salmonella typhimurium*, however, the chemical identity of the antimicrobial compound was suggested to be lactic acid, although in standard laboratory conditions (MRS medium), no active bacteriocin could be detected by researchers. This is in line with previous studies on *L. rhamnosus* anti-microbial activity [9]. To validate the bacteriocin production capacity as an adaptive factor in LGG we analysed the presence and expression of the bacteriocin genes using bioinformatic tools. The presence of bacteriocin genes in the genome of *L. rhamnosus* GG was reported earlier which stated the presence of a 8.7-kb putative type IIb bacteriocin operon (*LGG_02385- LGG_02392*) that encoded the various components required for bacteriocin synthesis, including the export protein, ABC/C39- type peptidase, 2-component signal transduction system, immunity protein and bacteriocin [10]. Therefore, we started with a detailed sequence analysis of bacteriocin genes of *L. rhamnosus* GG using BLASTp and BAGEL program as well as published literature [11, 12]. By organizing the cluster of the bacteriocin genes using Vector NTI software it could be concluded that the bacteriocin gene cluster of LGG contains the genes responsible for the biosynthesis, modification, regulation (*LGG_02397-LGG_02398*), immunity (*LGG_02390 & LGG_02395*) and transport of bacteriocin (*LGG_02385-LGG_02386*) outside the cell. Additionally it also contains the genes which are considered for the two component regulatory system (Histidine protein kinase *LGG_02387* and response regulator *LGG_02388*) and three component regulatory systems (Putative peptide pheromone *LRHM_02296*, histidine protein kinase *LGG_02387* and response regulator *LGG_02388*).

BLAST Protein Analysis: Protein blast of bacteriocin genes of LGG was performed to identify the sequence homology and genetic orientation of the bacteriocin genes with the related *Lactobacillus rhamnosus* strains. BLASTp results (Table 3) showed that that *L. rhamnosus* GG contained two genes (*LGG_02391* and *LGG_02392)* which were predicted to be for bacteriocin. Gene loci *LGG_02391* and *LGG_02392* shows about 100% similarity with related *L. rhamnosus* strains whereas *LGG_02392* shows 45% of homology with the well documented bacteriocin producing strain *Lactobacillus plantarum*. The *LGG_02386* that is believed to be the bacteriocin export protein share strong sequence similarities. It shares (59%) with *L. plantarum*, (57%) with *L. acidophilus* [13], (58%) with *L. salivarius* [14] which are able to produce class IIb bacteriocin. *The LGG_02400* and *LGG_02380* which are predicted to be a prebacteriocin showed 100% similarity with *L. rhamnosus* HN001.

BAGEL Program Analysis: BAGEL was applied to check the bacteriocin production by LGG. Detection of Open Reading Frame (ORF) makes BAGEL independent of GenBank annotations and thus prevents the oversight of small non-conserved ORFs (The most probable candidates for bacteriocin genes), which are omitted from many genome annotations. BAGEL predicted the presence of Class IIb bacteriocin genes (*LGG_02391-LGG_02392*), having a score of 175, high enough to annotate them as putative bacteriocin genes. According to Eijsink *et al.* [15], the genes encoding the two peptides of Class IIb bacteriocins are always next to each other on the same operon, so that the two peptides are produced in approximately equal amounts. On the basis of this information and genetic orientation of the bacteriocin gene cluster of LGG done by BLASTp and BAGEL it could be predicted that the genes that encode the two

Table 3: BLAST analysis of Lactobacillus rhamnosus GG bacteriocin genes with closely related Lactobacillus species

Genes of LGG [10]	Genes of LGG GG[33]	Predicted encoded function	Organismsharing homology	% Similarity
LGG_02380	LRHM_2289	Prebacteriocin	L. rhamnosus HN001	95%
LGG_02385	LRHM_2294	Bacteriocin ABC transporter permease	L. rhamnosusLMS2-1	98%
LGG_02386	LRHM_2295	Bacteriocin export ABC transporter	L. rhamnosusLMS2-1	99%
-	LRHM_2296	Putative peptide pheromone	L. rhamnosusLMS2-1	100%
LGG_02387	LRHM_2297	Two component sensor histidine kinase	L.rhamnosusLMS2-1	99%
LGG_02388	LRHM_2298	Two component response regulator, bacteriocin	L. rhamnosusLMS2-1	97%
LGG_02390	LRHM_2301	Bacteriocin immunity protein	L. rhamnosusLMS2-1	95%
LGG_02391	LRHM_2302	Hypothetical protein, Protein with bacteriocin type signal sequence, alpha unit	L. rhamnosus HN001	100%
LGG_02392	LRHM_2304	Hypothetical protein, Protein with bacteriocin type signal sequence, beta unit	L. rhamnosusLMS2-1	98%
LGG_02395	LRHM_2307	Immunity protein PlnI membrane bound protease CAAX family	L.rhamnosusLMS2-1	95%
LGG_02400	LRHM_2312	Prebacteriocin	L. rhamnosus HN001	100%

peptides might be *LGG_02391*and *LGG_02392*. The suggestion should be validated by further experimental evidence supported by expression studies of the bacteriocin genes of LGG.

Prediction of Promoters Using Fruit Fly Promoter Prediction Tool: Based on the gene orientation NCBI database (Pubmed) and use of the promoter prediction tools, there was a possibility of one promoter for the bacteriocin ABC transporter *LGG_02385-02386* before the gene, possibility of three promoters for immunity proteins *LGG_02390* and two promoters for *LGG_02395*, before and between the gene. Due to high level of conservation of the ABC transporters of class II bacteriocins, we went ahead for constructing the promoter probe construct for ABC transporter genes.

Construction of the TCR and Ery R CMPG 5340 Reporter Strain: For the mice experiments, a LGG control strain was constructed that could be used for the analysis of LGG wild type in competition with selected mutants and for the RIVET analysis as a reporter strain. The TcR marker tet (M) was first amplified from *L. plantarum* MD5057 [16] via a PCR reaction with primers Pro-221 and Pro-222. The DNA fragment containing the gene encoding the TcR marker was then blunted and ligated between the *loxP* sites of the plasmid pNZ7108 [17], which had been digested with restriction enzymes *Swa* I and *Nhe* I to remove the TcR marker gene already present in the plasmid. We replaced this TcR marker, since it was not functional in LGG. A RifR derivative of CMPG5340 was also obtained by serial culturing in increasing concentrations of Rif (up to 50µg/ml) to facilitate isolation of this strain in subsequent experiments from fecal samples of mice that also contain endogenous lactobacilli. Strains CMPG5340 and CMPG5340-Rif R were used as wild type control strain in the mice experiments.

Construction of the Promoter Probe Plasmid pCMPG5350: We first tested whether the pNZ7125 plasmid could be used as a promoter probe plasmid for the RIVET analysis in LGG. Unfortunately, this plasmid showed to be unstable in LGG, generating CmR colonies that do not show the correct replication of pNZ7125.Therefore we searched for other low copy theta replicating, preferentially, *E. coli-Lactobacilli* shuttle vectors that could be used to construct a promoter probe plasmid in LGG. Various plasmids were tested for their transformation and replication efficiency in LGG, i.e. pMEC10 [18], pTRKH2 and pTRKl2 [19], pRV566 [20], plAâ5 and plAâ8 [21] and pSIP409 [22]; pRV566, pTRKH2 and pTRKl2 could be transferred with a comparable efficiency to the plasmid pLAB1301 [23]. However for the multiple cloning steps required for the construction of the promoter probe plasmid, plasmid pLAB1301 [23] showed to be the best choice, although this plasmid possesses an ó- mechanism of replication and is less stably maintained without antibiotic selection. To be compatible with the EryR-TcR reporter strain CMPG 5340, the EryR marker of pLAB 1301 was removed by a restriction digest with *SnaB* I and *Sma* I and replaced by the boosted CmR marker, encoded by a *Dra* I-*Sma* I DNA fragment from plasmid pGIZ850 [24]. Subsequently, a PCR fragment containing the promoterless *cre* gene (Including the terminator) from plasmid pNZ7125 was amplified with primers Pro-0521 and Pro-0522 and cloned into the *Eco RI* site from the CmR pLAB 1301- derived vector described above. This resulted in the promoter probe plasmid pCMPG5350 (Fig. 2) used in these studies.

RIVET Animal Experiments of Promoter Probe Construct of ABC Transporter Genes of *L. Rhamnosus* GG: RIVET studies in murine model were carried out to check the presence of the putative encoding bacteriocin gene and also to check its role in their survival and adaptation mechanism inside the gastrointestinal tract. It was previously described by Bron *et al.* that the promoter of the *plnI* gene of *L. plantarum* encoding a plantaricin immunity protein, is specifically induced inside the murine GIT. Therefore, we wanted to investigate whether the bacteriocin genes of *L. rhamnosus* GG are also induced inside the murine GIT. To study the promoters that are specifically active *In vivo* and not *in vitro* is *In vivo* expression technology (IVET). RIVET has been used in several Gram-negative and Gram-positive bacteria to identify promoters and/or genes that are induced *In vivo* [25- 28]. The major advantages of RIVET over other strategies include the detection of transient gene induction in a small number of cells. RIVET also allows identification of niche-regulated genes expressed at different levels [29]. First, a promoter probe construct was made as described in the Materials and Methods section. This construct pCMPG5367 harbors the promoter of the *LGG_02385* and *LGG_02386* genes, encoding bacteriocin ABC transporter and bacteriocin export ABC transporter, ligated upstream of a *cre* gene that encodes a Cre resolvase in plasmid pCMPG5350 (Fig. 2). This construct was electroporated to the reporter strain CMPG5340-RifR that harbors chromosomally located *loxP-ery-loxP* cassette. Promoter activity of the bacteriocin genes was thus monitored by the Cre/Lox reporter system, which was previously implemented in *L. rhamnosus* GG for *lux S* knock out mutant for biofilm formation by Lebeer *et al.* [29]. The activity of this promoter was first checked *in vitro,* after overnight growth for 10 generations in MRS broth. Replica plating on MRS plates with 5 µg/ml of chloramphenicol and 10 µg/ml of tetracycline revealed that only 0.5% of the colonies are tetracycline sensitive, which showed that the expression of the promoter pCMPG5367 was very low and only in few cells (Stochastic gene expression). Stochastic gene expression occurs when transcriptional regulators are present at very low concentrations, so that binding and release of regulators from their binding sites becomes probabilistic. In contrast, the promoter of the *dlt* operon, encoding genes for D-alanylation of lipoteichoic acid essential for cell wall integrity, showed 100% promoter activity in MRS medium, which is indicative of a constitutive promoter [30]. Subsequently, the promoter activity of the bacteriocin ABC transporter genes of *L. rhamnosus* GG was tested *In vivo* in the murine GIT. Hereto, the same *L. rhamnosus* GG-derivative strain, CMPG5340-RifR containing pCMPG5367, was administered to two BALB/C mice by intragastric administration on 2 consecutive days. The fecal samples of different time points were replica plated on 5 µg/ml of chloramphenicol and 10 µg/ml of tetracycline. Only limited promoter activity could be detected: 0.2% of the colonies from the 48 h time point were TcS when confirmed with colony PCR (Table 4).

As a consequence, it is difficult to say whether the bacteriocin genes *LGG_ 02385-LGG_02386* are induced *In vivo.* A similar observation was seen in *L. casei* strains (to which *L. rhamnosus* GG is highly related), that showed a low rate of cell division in the murine GIT [31]. It is known that one cell division is needed for each Cre-mediated excision. This means that because of low rate of cell division the expression of the ABC transporter gene inside the murine model is low and so it gave low number of transformants. This result is similar to the results obtained in the microarray studies carried out during this study. Down-regulation of ABC transporter genes was found under bile conditions and also under different medium conditions like using whey medium and sugarless medium. At the same time expression of these genes were upregulated during the exponential growth phases and by using prebiotics like Fructo Oligo Saccharide. *L. plantarum* showed induction of the *plnI* gene encoding a bacteriocin immunity protein in the GIT [17], while the promoter of the ABC transporter genes was tested here for *L. rhamnosus* GG. This study suggests that in addition to direct competition between intestinal bacteria for adhesion, the rate of growth and division, or generation time, determines the ability of probiotics to colonize and persist on mucosal surfaces. These are factors that will also have an impact on probiotic efficacy and thus such factors should be included in the selection criteria for future probiotics. Despite of this low expression of this construct LGG is considered as a well documented probiotic strain as it has largest number of proven health benefits.

Therefore, it will be better to repeat the mice experiments by making different promoter probe constructs for genes like immunity protein (*LGG_02390* & *LGG_02395*) increasing the residence time of the *L. rhamnosus* GG. Prebiotics enhance survival and prolong retention of specific probiotic inocula in an *In vivo* murine model. So prebiotics like FOS which up-regulates the expression of this promoter (Seen in microarray studies) can be used to increase the expression of this promoter probe construct pCMPG5367 in murine model.

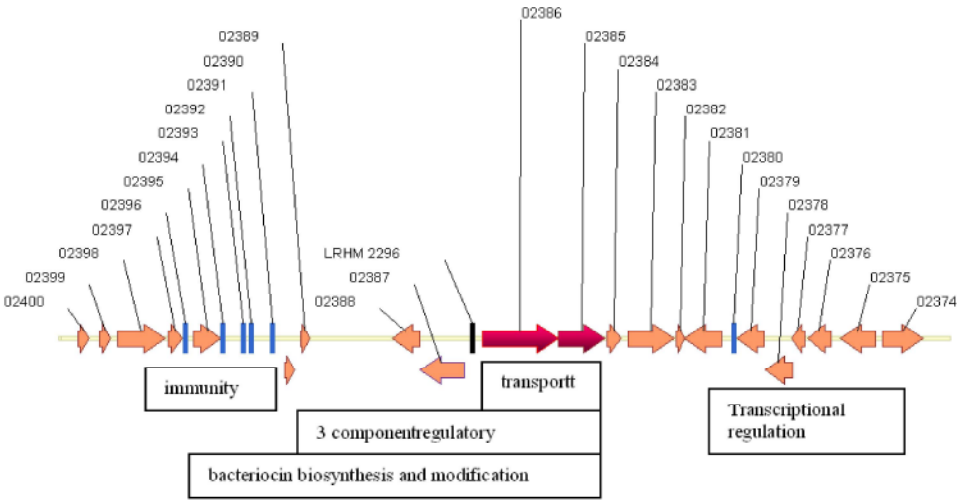

Fig. 2: Arrangement of the bacteriocin genes by Vector NTI software

Table 4: Percent of Tetracyline sensitive colonies during in vitro and in vivo RIVET experiments

Time point (hrs)	Total colonies	Tc^s (tetracycline sensitive colonies)	% of Tc^s
In vitro (48h)	5	5	0.5
In vitro Murine model (6h)	0	0	0
In vitro Murine model (24h)	0	0	0
In vitro Murine model (32h)	0	0	0
In vitro Murine model (48h)	12	2	0.2

CONCLUSION

One of the clinically best documented probiotic strains is *Lactobacillus rhamnosus* GG (LGG). Until now, studies of LGG have mainly focused on the description of a plethora of its health benefits. However to advance the field, molecular knowledge on the survival and persistence of LGG in the host (Adaptation factors) and the exertion of its beneficial effects at its site of action (Probiotic factors) is required to substantiate LGG's mode of action underlying its reported health promoting effects. By constructing a promoter probe construct pCMPG5367 that contains a promoter for the ABC transporter genes, it was demonstrated that this construct showed very low induction of the promoter by *Cre* mediated excision, because of very low rate of cell division. Additionally the bacteriocin gene cluster was identified and annotated by BLAST and BAGEL bioinformatic analysis which encoded various components required for bacterioicn synthesis, including the export protein, ABC/C39- type peptidase, 2-component signal transduction system, immunity protein and bacteriocin. The predicted bacteriocin from strain GG consisted of 2 short peptides both containing the bacteriocin type II double glycine leader motif (*LGG_02391-LGG_02392*) required for C39 peptidase mediated recognition and also *LGG_02400* which is predicted to be a class IIa bacteriocin. These, results support that bacteriocin may serve as an adaptation factors for the survival and persistence of *Lactobacillus rhamnosus* GG inside the gastrointestinal tract of mice.

ACKNOWLEDGEMENTS

We are thankful to Prof Jos Vanderleyden, Prof Sigrid Dekeersmaecker, Dr.Sarah Lebeer, Tina Verhoeven, David Decoster, from Katholeikke University of Leuven, for guiding the first author in her research work and providing lab space to complete this work. This study was supported by Erasmus Mundus Exchange Programme, EMECW15.

REFERENCES

1. Foster, J.A. and K.A. McVey Neufeld, 2013. Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci, 36: 305-312.
2. Doron, S., D.R. Snydman and S.L. Gorbach, 2005. Lactobacillus GG: bacteriology and clinical applications. Gastroenterol. Clin. North. Am., 34: 483-498.
3. Guarino, A., F. Albano, S. Ashkenazi, D. Gendrel, J.H. Hoekstra, R. Shamir and H. Szajewska, 2008. European Society for Paediatric Gastroenterology, Hepatology and Nutrition/European Society for Paediatric Infectious Diseases evidence-based guidelines for the management of acute gastroenteritis in children in Europe, Journal of Pediatric Gastroenterology and Nutrition, 46: 619-621.

4. Kalliomäki, M., S. Salminen, H. Arvilommi, P. Kero, P. Koskinen and E. Isolauri, 2001. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet, 357: 1076-1079.
5. Zocco, M.A., L.Z. Dal Verme, F. Cremonini, A. C. Piscaglia, E.C. Nista, M. Candelli, M. Novi, D. Rigante, I.A. Cazzato, V. Ojetti, A. Armuzzi, G. Gasbarrini and A. Gasbarrini, 2006. Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis. Alimentary Pharmacology and Therapeutics, 23: 1567-1574.
6. Silva, M., N.V. Jacobus, C. Deneke and S.L. Gorbach, 1987. Antimicrobial substance from a human Lactobacillus strain, Antimicrob. Agents Chemother. 31: 1231-1233.
7. Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning A Laboratory Manual. Book 3. Second Edition. Cold Spring Harbor Laboratory Press, pp: 1659.
8. De Keersmaecker, S.C., T.L. Verhoeven, J. Desair, K. Marchal J. Vanderleyden and I. Nagy, 2006. Strong antimicrobial activity of Lactobacillus rhamnosus GG against Salmonella typhimurium is due to accumulation of lactic acid, FEMS Microbiol Lett., 259: 89-96.
9. Lehto, E.M. and S.J. Salminen, 1997. Inhibition of Salmonella typhimurium adhesion to Caco-2 cell cultures by Lactobacillus strain GG spent culture supernate: only a pH effect? FEMS Immunol. Med. Microbiol., 18: 125-132.
10. Kankainen, M., L. Paulin, S. Tynkkynen, I. Von Ossowski, J. Reunanen, P. Partanen, R. Satokari, S. Vesterlund, A. P. Hendrickx, S. Lebeer, S.C. De Keersmaecker, J. Vanderleyden and T. Hämäläinen, S. Laukkanen, N. Salovuori, J. Ritari, E. Alatalo, R. Korpela, T. Mattila-Sandholm, A. Lassig, K. Hatakka, K.T. Kinnunen, H. Karjalainen, M. Saxelin, K. Laakso, A. Surakka, A. Palva, T. Salusjärvi, P. Auvinen and W.M. De Vos, 2009. Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein, Proc. Natl. Acad. Sci. USA, 106: 17193-17198.
11. Diep, D.B., O. Johnsborg, P.A. Risøen and I.F. Nes, 2001. Evidence for dual functionality of the operon plnABCD in the regulation of bacteriocin production in Lactobacillus plantarum, Mol. Microbiol., 41: 633-644.
12. Mathiesen, G., H.M. Namløs, P.A. Risøen, L. Axelsson and V.G. Eijsink, 2004. Use of bacteriocin promoters for gene expression in Lactobacillus plantarum C11, J. Appl. Microbiol., 96: 819-827.
13. Dobson, A.E., R.B. Sanozky-Dawes and T.R. Klaenhammer, 2007. Identification of an operon and inducing peptide involved in the production of lactacin B by Lactobacillus acidophilus, J Appl. Microbiol., 103: 1766-1778.
14. Corr, S.C., Y. Li, C.U. Riedel, P.W. O'Toole, C. Hill and C.G.M. Gahan, 2007. Bacteriocin production as a mechanism for the antfinfective activity of Lactobacillus salivarius UCC118, Proc Natl acad sci USA, 104: 7617-7621.
15. Eijsink, V.G., L. Axelsson, D.B. Diep, L.S. Havarstein, H. Holo and I.F. Nes, 2002. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication, Antonie Van Leeuwenhoek, 81: 639-654.
16. Danielsen, M., 2002. Characterization of the tetracycline resistance plasmid pMD5057 from Lactobacillus plantarum 5057 reveals a composite structure, Plasmid, 48: 98-103.
17. Bron, P.A., C. Grangette, A. Mercenier, W.M. De Vos and M. Kleerebezem, 2004. Identification of Lactobacillus plantarum genes that are induced in the gastrointestinal tract of mice, J. Bacteriol, 186: 5721-5729.
18. Pavan, S., P. Hols, J. Delcour, M.C. Geoffroy, C. Grangette, M. Kleerebezem and A. Mercenier, 2000. Adaptation of the nisin-controlled expression system in Lactobacillus plantarum: a tool to study *In vivo* biological effects, Appl. Environ. Microbiol, 66: 4427-4432.
19. O'Sullivan, D.J. and T.R. Klaenhammer, 1993. High- and low-copy number Lactococcus shuttle cloning vectors with features for clone screening, Gene, 137: 227-231.
20. Alpert, C.A., A.M. Crutz-Le Coq, C. Malleret and M. Zagoree, 2003. Characterization of a theta-type plasmid from Lactobacillus sakei: A potential basis for low-copy-number vectors in Lactobacilli, Appl. Environ. Microbiol, 69: 5574-5584.
21. Isabel Pérez-Arellano, Manuel Zúñiga and Gaspar Pérez-Martínez1, 2001. Construction of Compatible Wide-Host-Range Shuttle Vectors for Lactic Acid Bacteria and Escherichia coli, Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Apartado de Correos 73, 46100 Burjassot, Valencia, Spain, Plasmid, 46: 106-116.
22. Sørvig, E., G. Mathiesen, K. Naterstad, V.G. Eijsink and L. Axelsson, 2005. High-level, inducible gene expression in Lactobacillus sakei and Lactobacillus plantarum using versatile expression vectors, Microbiology, 151: 2439-2449.

23. Josson, K., T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau and J. Mahillon, 1989. Characterization of a gram-positive broad-host-range plasmid isolated from Lactobacillus hilgardii, Plasmid, 21: 9-20.
24. Louvain-la-Neuve, Biochimie et Génétique Moléculaire Bactérienne, Institut des Sciences de la Vie, Université´ Catholique de Louvain, Place Croix du Sud 5/6, B-1348 Louvain-la-Neuve, Belgium,1 and Danisco France SAS, BP 10, 86220 Dange´-Saint-Romain, France2.
25. Lowe, A.M., D.T. Beattie and R.L. Deresiewicz, 1998. Identification of novel staphylococcal virulence genes by *In vivo* expression technology, Mol. Microbiol., 27: 967-976.
26. Saviola, B., S.C. Woolwine and W.R. Bishai, 2003. Isolation of acid-inducible genes of Mycobacterium tuberculosis with the use of recombinase based *In vivo* expression technology, Infect. Immun, 71: 1379-1388.
27. Veal-Carr, W.L. and S. Stibitz, 2005. Demonstration of differential virulence gene promoter activation *In vivo* in Bordetella pertussis using RIVET, Mol. Microbiol., 55: 788-798.
28. Tamir-Ariel, D., N. Navon and S. Burdman, 2007. Identification of genes in Xanthomonas campestris pv. vesicatoria induced during its interaction with tomato, J. Bacteriol., 189: 6359-6371.
29. Sarah Lebeer, C., J. Sigrid, De Keersmaecker and Jos Vanderleyden, 2007. Functional Analysis of *luxS* in the Probiotic Strain Lactobacillus rhamnosus GG Reveals a Central Metabolic Role Important for Growth and Biofilm Formation, J. Bacteriol, 189(3): 860-871, doi: 10.1128/JB.01394-06.
30. Lee, Y.K., P.S. Ho, C.S. Low, H. Arvilommi and S. Salminen, 2004. Permanent colonization by Lactobacillus casei is hindered by the low rate of cell division in mouse gut, Appl. Environ. Microbiol, 70: 670-674.
31. Marco, M.L., R.S. Bongers, W.M. De Vos and M. Kleerebezem, 2007. Spatial and temporal expression of Lactobacillus plantarum genes in the gastrointestinal tracts of mice, Appl. Environ. Microbiol., 73: 124-132.
32. Perea Velez, M. Verhoeven, T.L.A. Draing, C. Von S. Aulock, M. Pfitzenmaier, A. Geyer, I. Lambrichts, C. Grangette, B. Pot, J. Vanderleyden and S.C.J. De Keersmaecker, 2007. Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG, Appl. Environ. Microbiol., 73: 3595-3604.
33. Morita, H., H. Toh, K. Oshima, M. Murakami, T. D. Taylor, S. Igimi and M. Hattori, 2009. Complete genome sequence of the probiotic Lactobacillus rhamnosus ATCC 53103, J. Bacteriol., 191(24): 7630-7631.