

## Identification of the Lactobacillus SLP domain that binds gastric mucin

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### 1. ABSTRACT

Surface layer proteins (SLPs) of lactobacillus bacteria have some structural regions responsible for adhesion to the intestinal epithelium. To identify the SLP and the smallest domain within the protein that is responsible for the adhesion of the bacterium to the

intestinal epithelium, *L. plantarum* strain CGMCC1258 was investigated in this study. Using bioinformatics and molecular techniques, we first identified and purified a novel protein, integral membrane protein-2 (IMP-2, 33-45 kDa) responsible for adhesion to gastric mucin. Truncated forms of IMP-2 were then constructed and expressed, and the amino acids from 515 to 575 (designated micro IMP,

MIMP) was identified as the smallest domain responsible for adhesion to gastric mucin. Competing assay was performed, which further confirmed the ability of MIMP to compete with enteroinvasive *E. coli* and enteropathogenic *E. coli* to adhere to cells of a normal colon cell line, NCM460. Furthermore, MIMP could mature dendritic cells. These findings set a foundation for further investigation on the role of MIMP in the treatment and prevention of inflammation-related diseases of the intestine.

## 2. INTRODUCTION

The population of viable microorganisms in the human gut exceeds the total number of somatic and germ cells in our body (1). There is growing evidence that those bacterial species closely related and adherent to the mucosa are more relevant to human health than those evacuated in the feces (2-3). In healthy individuals, there is a homeostasis between commensal microbiota and pathogenic bacteria in the intestines (4-5). Alterations in this homeostatic mechanism will result in an imbalanced ecological microenvironment and subsequent gut-barrier dysfunction (6-7). Moreover, the predominance of pathogenic bacteria and/or the excessive production of bacterial products, including endotoxins and antigens, may directly and indirectly initiate and amplify local and systemic inflammatory responses (8).

It has been established that the symbiotic bacteria of the genus *Lactobacillus* in the gut play a significant role in maintaining the homeostasis of the gut flora by adhering to and colonizing the intestinal mucosa where they compete with pathogenic bacteria (9-10). The interactions between *Lactobacillus* and the intestinal tract are carried out by adhesion of the bacteria to the epithelium (11-12). Furthermore, they also block the adhesion of other pathogenic bacteria, such as enteropathogenic *Escherichia coli* (EPEC) and enteroinvasive *E. coli* (EIEC).

Recently, particular attention has been paid to the bacterial surface composition, especially the surface layer proteins (SLPs), in studies investigating adhesion of the *Lactobacillus* bacteria to the intestinal epithelium (13-15). SLPs of *Lactobacillus* bacteria are ubiquitous cell envelope structures composed of numerous identical protein/glycoprotein subunits (16); SLPs exhibit an intrinsic ability to spontaneously form regular layers either in a solution or on a solid support under certain conditions (17). A few studies have demonstrated that SLPs play a critical role in the process of adhesion of the bacteria to the intestinal epithelium (14, 18-19). However, because SLPs have special hydrophilic and hydrophobic properties, and thus are difficult to purify (20), only a few studies (14, 19) have explored the role of SLPs in the adhesion process. Therefore, the roles of SLPs in the adhesive process, and in particular the subsequent activities such as potential protecting effect on the intestinal permeability and anti-inflammatory effect, remain poorly understood.

Our previous studies demonstrated that *L. plantarum* was able to prevent EIEC-induced damage to

colorectal adenocarcinoma cells, including transepithelial electrical resistance and dextran integrated intensity, as well as changes in the re-expression of tight junction (TJ) proteins, such as claudin-1, occludin, JAM-1 and ZO-1 (21); we also showed that oral *L. plantarum* treatment was able to restore hepatic barrier function in rats with obstructive jaundice by increasing mRNA and protein levels of TJ proteins (22) and prevents the up-regulation of adhesion molecule expression in IL-10 knock-out mice (23). Moreover, we noted that enteral feeding of *L. plantarum* attenuated disease severity and improved intestinal permeability and clinical outcomes in patients with acute pancreatitis (24). However, the particular bacterial protein and its exact mechanisms of action remain unknown.

Therefore, we carried out this study in an attempt to identify the protein and the smallest active domain within the protein from *L. plantarum* strain CGMCC1258 that is responsible for the adhesion of the bacterium to the intestinal epithelium. First, the integral membrane protein (IMP, GenBank Identifier 28378881), one kind of SLP in the *L. plantarum* strain was identified as having the ability to adhere to gastric mucin. Furthermore, the smallest functional domain within IMP, named micro IMP (MIMP), was identified and purified.

## 3. MATERIALS AND METHODS

### 3.1. Protein expression system and reagents

The bacterial strains and plasmids used in this study and their sources are listed in Table 1. *L. plantarum* strain CGMCC1258 was provided by the Institute of Biomedicine, Shanghai Jiao Da Only Company Ltd, Shanghai, China. EIEC strain ATCC 43893 (O124:NM) and EPEC ATCC43887 (O111:NM) were obtained from Shanghai Municipal Center for Disease Control and Prevention. *E. coli* strains DH10B and BL21 (DE3) were purchased from Novagen (Madison, WI, USA). Plasmid *pET32*, which was used for plasmid construction, gene cloning and protein expression, was from Novagen. NCM460 cell line, a normal colon cell line, was purchased from INCELL Corporation (San Antonio, TX, USA). Specific pathogen-free New Zealand white rabbits (*Oryctolagus cuniculus*) were from the Animal Facility at Shanghai Jiao Tong University School of Medicine.

The restriction endonucleases (*Bgl*II, *Xho*I and *Hind*III), T4 DNA ligase, Taq DNA polymerase, isopropylthio-beta-D-galactoside (IPTG) and the pre-stained protein marker were purchased from MBI (Fermentas, New England Biolabs, MA, USA). Kits for rapid extraction of plasmid DNA in *E. coli* cells were purchased from Zhongshan Biological Engineering Company (Beijing, China) or Promega, (Madison, WI, USA). Agarose gel DNA purification kit was provided by Zhongshan Biological Engineering Company. The biotinylated protein ladder was from Cell Signaling (Dallas, TX, USA). Partially purified mucin from porcine stomach, horseradish peroxidase (HRP), ethylenediaminetetraacetic acid (EDTA) solution and Dulbecco's modified Eagle's medium (DMEM) were from Sigma-Aldrich (Steinheim,

**Table 1.** Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
<i>Lactobacillus plantarum</i>	CGMCC 1258	CGMCC
Enteroinvasive <i>E. coli</i>	O124:NM, ATCC 43893	ATCC
Enteropathogenic <i>E. coli</i>	O111:NM, ATCC 43887	ATCC
<i>E. coli</i> DH10B	F- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ-	Invitrogen
<i>E. coli</i> BL21(DE3)	F-ompT hsdSB (rB- mB-) gal dcm (DE3)	Novagen
Plasmids		
pET32	Ampr, <i>E. coli</i> expression vector	Novagen
PATRI455-755	Ampr, pET32(Tag-linker- IMP455-755)	This study
PATRI455-575	Ampr, pET32(Tag-linker- IMP455-575)	This study
PATRI455-635	Ampr, pET32(Tag-linker- IMP455-635)	This study
PATRI455-695	Ampr, pET32(Tag-linker- IMP455-695)	This study
PATRI635-755	Ampr, pET32(Tag-linker- IMP635-755)	This study
PATRI575-755	Ampr, pET32(Tag-linker- IMP575-755)	This study
PATRI515-755	Ampr, pET32(Tag-linker- IMP515-755)	This study
PATRI515-575	Ampr, pET32(Tag-linker- IMP515-575)	This study

Ampr, resistance to kanamycin. Sequence of the designed tag: MTVNTDYHGGVNHVGANGYDNNLDRMAGQGDSIMSDGISVLYQFMTLFTDLAQGKYDQMKAKADRARMTVNTDY HGGVNHVGANGYDNNLDRMAGQGDSIMSDGISVLYQFMTLFTDLAQGKYDQMKAKADRARNSSQVANQIDAIKAF KKAGDKGDLPEVLKYLDRHNINITYQDDGKNATSDIDQYLKSIGHPDGKGLDKGELDVIKGALETDSGRSSDFVTQAQ LQIQKTMQSYNVCVSLNSMQTLAEMNKSIQNR

Germany). De Man-Rogosa-Sharpe (MRS) broth was from Difco Laboratories (Detroit, MI, USA). The HRP-conjugated anti-rabbit IgG secondary antibody was from Santa Cruz (CA, USA). PVDF membrane was from Millipore (Bedford, MA, USA). iPrimer synthesis and sequencing was performed by Sangon (Shanghai, China). Fetal bovine serum was from Gibco (Carlsbad, California, USA). Oligonucleotides (Oligomer, Helsinki, Finland) used in this study are listed in Table 2. DNA sequencing kit for BigDye Terminator cycle sequencing was from Applied Biosystems (Foster City, CA, USA) and the QIAquick PCR purification kit was from Qiagen (Hamburg, Germany).

### 3.2. Culture of *L. plantarum* and isolation of SLPs

*L. plantarum* CGMCC1258 was inoculated in fresh 5% MRS broth at 37°C for 24-h. The bacterial culture was centrifuged (×3 500g) at 4°C for 20 min, and then the supernatant was discarded. 2M guanidine-HCl (Sigma-Aldrich) was added to the solution and incubated for 3-h. The suspension was centrifuged (×6000g) at 4°C for 20 min. The supernatant was dialyzed in 0.01M phosphate buffered saline (PBS, pH 7.4) overnight and then centrifuged (×40000 g) at 4°C for 60 min, after which the supernatant was removed. The pellet was dissolved in 100 μL 0.01M PBS, incubated at 70°C for 30 min and centrifuged (×16000g) at 4°C for 20 min. The supernatant was used for the preliminary screening of SLP adhering to gastric mucin by SDS-PAGE and Western blotting.

### 3.3. Labeling of porcine gastric mucin with HRP

Partially purified mucin from porcine stomach was labeled with HRP as described previously (25). Briefly, 8 mg HRP was dissolved in 2 mL distilled water and then 400 μL of freshly prepared sodium periodate (0.1M) was added. The mixture was stirred gently for 20 min at room temperature and then dialyzed overnight at 4°C against 0.001M acetate buffer (pH 4.4). Sodium carbonate buffer (0.1M, pH 9.5; 20 μL) was added to raise the pH to 9.0-9.5, and 1 mL of treated HRP was immediately mixed with 1 mL of gastric mucin (4 mg/mL in sodium carbonate buffer).

The mixture was held at room temperature for 2-h with occasional stirring. After 100 μL freshly prepared sodium borohydride solution (4 mg/mL) was added to reduce any free enzyme, the mixture was dialyzed against borate buffer (0.1 M, pH 7.4). Finally, the labeled gastric mucin was mixed with equal volumes of 80% glycerol and stored at -20°C until use (19).

### 3.4. Identification and verification of proteins and peptides responsible for adhesion to the gastric mucin

#### 3.4.1. Preliminary screening of SLP(s) adhering to gastric mucin by SDS-PAGE and Western blotting

SDS-PAGE was performed with a discontinuous gradient 5% (w/v) stacking gel and a 10% (w/v) separating gel in a Miniprotean II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant of *L. plantarum* was mixed with loading buffer containing SDS and beta-mercaptoethanol, boiled for 3 min, centrifuged, and loaded onto the SDS-PAGE gels for separation. Gels were stained with Coomassie brilliant blue R-250, and the molecular weight of the SLP was determined by comparing its electrophoresis mobility with marker proteins. For Western blotting, the gels were transferred to a PVDF membrane in a semidry electroblotter (Bio-Rad Laboratories) for 120 min at 100 V. The membrane was washed three times (20 min each) with PBS containing 0.1% Tween-20 (PBS-T buffer), and then treated with HRP-labeled gastric mucin (diluted 1:600, about 0.035 μmol/L) in PBS-T buffer for 4 hr at 4°C. The membrane was washed three times (60 min each time) with PBS-T buffer. The SLP was tested by ECL kit (Pierce, Rockford, IL, USA) according to manufacturer's instructions.

#### 3.4.2. Detection of the protein adhering to the gastric mucin by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

The target protein bands were excised from the SDS-PAGE gel and analyzed by LC-MS/MS. The MS result was initially analyzed with the SEQUEST software (Thermo Fisher Scientific, San Jose, CA, USA), and then

**Table 2.** Oligonucleotides used in this study

Gene	primer
PO	atcagatctgATGGCAAAGATGATTGAAGC atctcgcagCTATTCGACGTTGAACCT
GBP	atcagatctgTTGAAATTTAGAGATGATAT atctcgcagTTACTTGTTCATTTTACGCT
CD	atcagatctgGTGGGTGTGATGGCCATTTT atctcgcagTCAATTATTCTGATTCTTGA
IMP1	atcagatctgTTGAAGACAGTACGGAAGAT atctcgcagAATCGCAGCAAGATCAACGC
IMP2	atcagatctgAATACTAATTCTAATAGTAC atctcgcagAAGATAGAGTCCGTATTTC
IMP3	atcagatctgAAGCCAACCGTTACCGGAAA atctcgcagTTTATGCTCGGTGTGTGAT
Truncations	
IMP455–755	atcagatctg AATACTAATTCTAATAGTAC atctcgcag AAGATAGAGTCCGTATTTC
IMP455–575	atcAAGCTTAATACTAATTCTAATAGTAC atctcgcagAGTTTTCATCTTAACAACCT
IMP455–635	atcAAGCTTAATACTAATTCTAATAGTAC atctcgcagAACCTGCTGCGTAGTGACTG
IMP455–695	atcAAGCTTAATACTAATTCTAATAGTAC atctcgcagCAAACTTTCTCGTTGTTCGG
IMP635–755	atcAAGCTTAGTGTTAAGACTGTCAA atctcgcagAAGATAGAGTCCGTATTTC
IMP575–755	atcAAGCTTACTGGCGAACCAGAGATTT atctcgcagAAGATAGAGTCCGTATTTC
IMP515–755	atcAAGCTTTTTTCAGTTCAAAATGGTTA atctcgcagAAGATAGAGTCCGTATTTC
MIMP	atcAAGCTTTTTTCAGTTCAAAATGGTTA atctcgcagAGTTTTCATCTTAACAACCT

PO, pyruvate oxidase; CD, cell division initiation protein FtsQ; GBP, GTP-binding protein TypA; IMP, integral membrane protein; MIMP, micro IMP.

with the Trans-proteomics Pipeline software (TPP, Center for Systems Biology, Institute for Systems Biology, Seattle, WA, USA) and FASTA software to further identify the related protein (26-27).

### 3.5. Identification of the protein adhering to gastric mucin by cloning and expression of genes encoding the putative target proteins

#### 3.5.1. Cloning of genes encoding the potential target proteins in *E. coli*

LC-MS/MS indicated that the target protein could be the GTP-binding protein TypA, pyruvate oxidase, cell division initiation protein FtsQ or integral membrane protein. Therefore, the corresponding genes encoding these putative proteins were selected for cloning and expression. IMP (containing 1022 amino acids), which has a transmembrane region according to the TMHMM software from ExPASy (<http://cn.expasy.org/>), was fractionally expressed as three sections, namely, IMP-1 (i.e. IMP1-454), IMP-2 (i.e. IMP455-755) and IMP-3 (i.e. IMP756-1022).

Briefly, the genomic DNA of *L. plantarum* CGMCC1258 was isolated by using QIAmp DNA Mini kit (QIAGEN Ltd., Crawley, United Kingdom). The primers were designed based on the DNA sequences of the genes in GenBank (Table 2) to amplify the target genes. PCR was carried out in a total volume in 100 µL containing 5 µL genomic DNA, 20pmol of each primer, 20µmol dNTPs, 1 × *Pyrococcus furiosus* (pfu) reaction buffer and 5U pfu DNA polymerase. PCR was performed under the following conditions: the genomic DNA was denatured at 94°C for 3 min followed by 30 amplification cycles (94°C for 30 s, 56°C for 30 s, and 72°C for 2 min) and followed by a final

extension at 72°C for 15 min. The PCR products were identified by 3% agarose gel electrophoresis.

The PCR products were recovered with DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). After digestion with BglII and XhoI, the PCR products were inserted into linearized pET32 and ligated by T4 DNA ligase to construct a prokaryotic recombinant expression plasmid. The plasmid containing the target gene was transfected into competent *E. coli* DH10B. The entire transformation mixture was plated on culture plates and further screened in the Terrific Broth (TB) medium. The recombinant plasmids of positive clones were extracted by the kits for rapid extraction of plasmid DNA (Promega, Madison, WI, USA), screened by PCR and then sequenced. The sequencing results were compared with the online program BLAST (Table 3).

#### 3.5.2. Expression of pET32-target genes in *E. coli* and purification of the expressed proteins

The recombinant plasmids containing different genes were extracted from *E. coli* DH10B and transfected in competent *E. coli* BL21 (DE3), according to the pET System Manual (Novagen). Briefly, the colonies were inoculated into TB medium containing 100 µg/mL ampicillin and 25 µg/mL kanamycin at 37°C with shaking (200 rpm). IPTG was added (final concentration: 1mM) into the medium after the optical density (OD) value at 600 nm reached approximately 0.6, and then the bacterial cells were incubated at 37°C for 3-h to induce the expression of the recombinant protein. After induction, the bacterial cells were harvested by centrifugation (×800 g) for 5 min and sonicated on ice. The supernatant and pellet were then separated by centrifugation (×800 g for 5 min). The

**Table 3.** Expression of putative target proteins

Protein	PI	Genbank number	Expression
Pyruvate oxidase	5.00	ABC47131.1	Full length
Cell division initiation protein FtsQ	10.32	NP_785691.1	Full length
GTP-binding protein TypA	5.12	NP_785653.1	Full length
Integral membrane protein	10.02	NP_785773.1	Segments

PI, Predicted isoelectric points.

separated supernatant or pellet were added to 1 ml of His-Tag fusion protein purification beads (Amersham Biosciences, Uppsala, Sweden), concussed for 30 min at 4°C according to the manufacturer's instructions. Then, His-tagged fusion proteins were dialyzed overnight at 4°C against PBS and concentrated by using Microcon 10 columns (Millipore) before storage at 4°C for up to 1 week (28). The protein content was quantitated by the BCA protein assay (Pierce, Rockford, IL, USA).

### 3.5.3. Confirmation of the ability of the target protein to adhere to the gastric mucin

The purified expressed proteins prepared above were loaded and run on paired 10% SDS-PAGE gels, as described above. One gel for each protein was stained with Coomassie brilliant blue and the other was transferred to a PVDF membrane in a semi-dry electroblotter. The membrane was treated with HRP-labeled gastric mucin and tested by ECL to identify the target protein, as described above.

### 3.5.4. Identification of the smallest domain within IMP-2 responsible for adhesion to the intestinal epithelium

Since IMP-2, known as IMP455-755, was identified as the protein responsible for adhesion to the gastric mucin by the above described methods, we carried out further experiments to identify the domain within IMP-2 that is responsible for its adhesion property.

Primary amino acid sequence analysis of IMP-2 with BLASTP was carried out to predict the domain responsible for its adhesion property. We attempted to search for the domain within IMP-2 responsible for its adhesion property by comparing the primary amino acid sequence of IMP-2 with those of other proteins with the similar structure. Amino acid sequence of IMP-2 was searched by using BLASTP with default parameters against NCBI non-redundant database ([http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE\\_TYPE=BlastDocs&DOC\\_TYPE=Download](http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download)). Then, a homology search for other proteins with the similar structure in the UniProt database was carried out by using BLASTP, and these proteins were selected for comparison in the primary amino acid sequences between IMP-2 and these proteins. Predicted isoelectric points of all proteins were obtained by ProtParam (<http://www.expasy.ch/tools/protparam.html>). Multiple Sequence Alignment analyses were performed by ClustalW using Gonnet as a comparison matrix (gap open penalty, 10; gap extension penalty, 0.2). The hydrophobicity was calculated according to Kyte and Doolittle with a window of seven amino acids.

### 3.5.5. Construction of plasmid vectors

The genomic DNA of *L. plantarum* CGMCC1258 was isolated as described above. The gene

encoding IMP-2 was amplified by PCR from the genomic DNA using the corresponding primer pairs (Table 2). The resulting PCR product was inserted into pET32, generating the plasmid PATRI455-755 encoding IMP-2 with a N-terminal His-tag (Table 1).

Three C-terminal truncations and three N-terminal truncations of IMP-2 were further constructed, each with a His-tag sequence at the N-terminus. The plasmid constructs are summarized in Table 1.

For cloning the three C-terminal truncations, primer pairs for IMP455-575 (encoding IMP455-575 domain), IMP455-635 (encoding IMP455-635 domain) and IMP455-695 (encoding IMP455-695 domain) (Table 2) were used for PCR amplification, with plasmid PATRI455-755 DNA as a template. The PCR products were then inserted into pET32. The resulting plasmids were named as PATRI455-575, PATRI455-635 and PATRI455-695 (Table 1).

Cloning of the three N-terminal truncations of IMP-2 was carried out with primer pairs for IMP635-755 (encoding IMP635-755 domain), IMP575-755 (encoding IMP575-755 domain) and IMP515-755 (encoding IMP515-755 domain) (Table 2) with PATRI455-755 DNA as a template. The resulting PCR products were inserted into pET32, generating plasmids, PATRI635-755, PATRI575-755 and PATRI515-755 (Table 1).

### 3.5.6. Expression and purification of the mature IMP-2 and its truncated forms encoded by the plasmids

Gene expression was carried out in *E. coli* DH10B transfected with each of the above seven recombinant plasmids, according to the pET System Manual (Novagen) and as described above. Briefly, IPTG at a concentration of 0.1 mM was added to the culture medium of competent *E. coli* (i.e. OD600 value of approximately 0.6) transfected with each of the seven expression plasmids. Incubation was continued for 3-5 h, depending on the protein to be expressed and purified. Thereafter, the recombinant mature IMP-2 and its truncated forms were purified with high performance liquid chromatography (HPLC) by passing through a porcine stomach mucin (PSM)-Sephrose 4B column which was equilibrated with 10 mM Tris-HCl, pH 8 containing 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 0.02% (w/v) NaN<sub>3</sub>. Unbound proteins were eluted and the adhesive protein was desorbed with 20 mM 1, 3-diaminopropane. The final fraction represented the purified adhesive protein (29).

### 3.5.7. Identification of the ability of recombinant IMP-2 forms to adhere to the gastric mucin by Western blotting with ECL

After purification, the fractions containing recombinant mature IMP-2 or each of its truncated forms

## Molecular identification of MIMP

**Table 4.** MIMP polyclonal anti-MIMP antibodies titer as measured by indirect ELISA

	Negative serum	Blank control	Polyclonal anti-MIMP antibodies					
Dilution	1/1000	1/1000	1/16k	1/64k	1/256k	1/512k	1/1024k	1/2048k
OD	0.023	0.021	2.772	2.635	2.58	2.218	1.799	1.186
SD	0.003	0.004	0.232	0.256	0.282	0.266	0.122	0.108

ELISA, enzyme-linked immunosorbent assay; OD, optical density; SD, standard deviation; Negative serum indicates the serum from a rabbit without MIMP immunization.

**Table 5.** Experimental grouping of NCM460 cells for competing adhesion assay

Group	Description
Control	Cell monolayers with no infection and no treatment
EIEC or EPEC	Cell monolayers infected by EIEC or EPEC
<i>L. plantarum</i> +EIEC or EPEC	Cell monolayers infected by EIEC and treated with <i>L. plantarum</i>
MIMP +EIEC or EPEC	Cell monolayers infected by EIEC or EPEC and treated with MIMP
Anti-MIMP+MIMP +EIEC or EPEC	Cell monolayers pre-incubated for 2 h with polyclonal anti-MIMP antibodies, then infected with EIEC or EPEC and treated with MIMP

were dialyzed overnight at 4°C against distilled water. Adherence of recombinant mature IMP-2 and its truncated forms was verified by 10% SDS-PAGE and Western blotting, as described above.

### 3.5.8. Expression and purification of MIMP

From the above experiments, the peptide IMP515-575 was identified as the smallest domain (i.e. micro IMP, or MIMP) that was responsible for adhesion to the gastric mucin. Therefore, the above techniques were used to construct the plasmid pET32 (Tag-linker-IMP515-575), to clone the gene encoding MIMP, and to express and purify MIMP. The purified recombinant MIMP was used for further characterization.

### 3.5.9. Preparation of polyclonal anti-MIMP antibodies and identification by Western blotting with ECL and enzyme-linked immunosorbent assay

The purified recombinant MIMP was used to immunize a rabbit (New Zealand white, adult, male *Oryctolagus cuniculus*) to generate the serum containing polyclonal antibodies against MIMP. Briefly, 0.3 ml (2 g/L) of the purified protein emulsified in complete Freud's adjuvant was injected intraperitoneally at multiple points on the back and groin (200 µg each point) for the first immunization, and half the dose in incomplete Freud's adjuvant was used for the booster immunization after two weeks, and repeated two more times. Fourteen days after the last booster, serum was collected and purified by affinity chromatography on 1 ml MIMP-Sepharose 4B column and stored at -20°C until use.

The polyclonal anti-MIMP antibodies were identified by Western blotting and enzyme-linked immunosorbent assay (ELISA). For Western blotting, 1, 10 and 100 pg of purified MIMP (2 µL) were mixed with 8 µL loading buffer (5×) and electrophoresed on a 10% SDS PAGE gel as described above. Gels were transferred to a PVDF membrane, which was then treated with polyclonal anti-MIMP antibodies (diluted 1:10000) and subsequently with HRP-conjugated goat anti-rabbit IgG antibody. The target protein was tested by using ECL according to the manufacturer's instructions. For ELISA identification, each of the wells of a polystyrene plate was coated with 100 µL (1 mg/L) purified MIMP overnight at 4°C. The plate was washed three times with PBS-T buffer, and blocked with 5% dry milk in PBS at a volume of 200 µL/well. Then, 100

µL different concentrations of diluted polyclonal anti-MIMP antibodies (Table 4) were added to designated wells; serum with IgG used at the identical concentration from a normal rabbit was used as a negative control. Each concentration was set up in duplicate, as well as the negative control group (37°C, 3-h incubation). After washing three times with PBS-T buffer, 100 µL HRP-conjugated anti-rabbit IgG antibody (diluted 1:5000 in PBS containing 1g/L BSA) was added. The plate was then washed three times with PBS-T buffer. Each well was spiked with 50 µL of o-phenylenediamine colored solution (Sigma-Aldrich) in dark for 10-15 min at room temperature, and then the reaction was stopped with 25 µL (2mol/L) ammonium sulfate. The OD value was determined by an ELISA reader (Bio-Tek Instruments, Inc., Winooski, Vt., USA) at 490nm. 3.5.10 Detection of IMP isolated and purified from *L. plantarum* by polyclonal anti-MIMP antibodies

To further confirm whether MIMP is the domain protein of IMP of *L. plantarum*, IMP isolated and purified directly from *L. plantarum* using HPLC as described above. Then, purified IMP was subjected to SDS-PAGE, and then identified by Western blotting as described above. The polyclonal anti-MIMP antibodies (diluted 1:600 in PBS-T buffer) were used as the primary antibodies, and HRP-conjugated anti-rabbit IgG antibody as the secondary antibody.

### 3.6. Confirmation of the ability of the purified MIMP to competitively adhere to intestinal epithelial cells

NCM460 cells grown on M3 base at 37°C in a 95% humid atmosphere, with 5% CO<sub>2</sub> for 72-h were washed three times in Hank's balanced solution (Life Technologies, San Diego, CA, USA) to remove the antibiotic medium. Then, the cells were cultured as monolayers (~1×10<sup>7</sup> for each monolayer) at 37°C in a 95% humid atmosphere, with 5% CO<sub>2</sub> and divided into 10 different triplicate experimental groups (Table 5). In EIEC and EPEC groups, 100 µL EIEC ATCC43893 (O124:NM) and EPEC ATCC43887 (O111:NM) of logarithmic growth phase 1.0×10<sup>8</sup>/mL were, respectively, added to the apical side of the cell culture insert for rapid infection of the monolayer for 3 hr of incubation, with an inoculation ratio of EIEC/EPEC to NCM460 cells of 100:1. The insert was placed in a 50-mL tube and centrifuged (×200 g for 4 min). In *L. plantarum* and MIMP groups, *L. plantarum* (100 µL

## Molecular identification of MIMP

of  $1.0 \times 10^8$ /mL) and MIMP (100  $\mu$ L of 0.1ng/mL) purified from the whole cell proteins of transfected *E. coli* were, respectively, added onto the monolayer of NCM460 cells simultaneously with EIEC/EPEC infection. In antibody groups, NCM460 cells were pre-incubated with the serum containing polyclonal anti-MIMP antibodies (100  $\mu$ L of dilution 1:5000) prepared as described above, prior to infection with EIEC or EPEC which was simultaneously incubated with MIMP. NCM460 cells cultured under the same conditions but without pre-incubation with the serum, infection of EIEC/EPEC and addition of *L. plantarum* or MIMP served as the control group.

After incubation of NCM460 cells for 24-h and trypsinization, the numbers of adhered bacteria and NCM460 cells were counted as colony forming units (CFU) by plating diluted bacterial suspensions on MRS or blood agar plates. NCM460 cells were counted by a Sysmex SE900 automatic cell counter (Toa Medical Electronics, Kobe, Japan). Adhesive rate %=(the number of bacteria adhering to the cells /the overall number of bacteria included)  $\times 100\%$ .

### 3.7 Flow cytometry for determining dendritic cell (DC) maturation

iDCs were obtained from the buffy coats of healthy donors, as described elsewhere (30). Briefly, human peripheral blood mononuclear cells were isolated by a Ficoll gradient. Monocytes were isolated by CD14 magnetic microbeads (MACS; Miltenyi Biotec, CA, USA) and differentiated into iDCs in the presence of IL-4 and human GM-CSF (500 and 800 U/mL, respectively). Then, iDCs were sub-cultured in Iscove's Modified Dulbecco's Medium, supplemented with 10% FBS, 500 U/mL IL-4, and 800 U/mL GM-CSF. In the experiments, AZN-D1, a neutralizing antibody against DC-SIGN,  $\text{Ca}^{2+}$  chelator EDTA, and polyclonal antibodies against MIMP were used in different groups where appropriate.

To determine the degree of maturation of DCs after exposure to *L. plantarum* or MIMP, expression of cell surface markers (including CD80, CD86 and MHC-II) were measured. Briefly, iDCs were cultured with *L. plantarum* (10 to 1,000 cfu per iDC) or MIMP (0.01 to 1  $\mu$ g/mL) with or without AZN-D1 (20  $\mu$ g/mL) or EDTA (10mM) at 37°C for 45 min, harvested, washed with PBS, stained with antibodies against CD80, CD86 or MHC-II at 4 °C for 1 h, and analyzed by FACS flow cytometry.

### 3.8. Statistical analysis

The results have been expressed as mean with standard deviation (SD). Statistical analyses were performed by using SPSS 11.0 systems (SPSS Inc. Chicago, IL, USA). Data were analyzed by one-way ANOVA on condition of homogeneity of variance and normal distribution of the variables. Dunnett-t test was used to carry out multiple comparison of experimental and control groups. A P value of  $<0.05$  was considered to be statistically significant.

## 4. RESULTS

### 4.1. Identification of IMP-2

#### 4.1.1. Detection of the SLP of *L. plantarum* adhering to the gastric mucin

SLPs were isolated using guanidine hydrochloride and ultracentrifugation; protein fractions were separated and detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, as described above. There were several bands of interest with molecular weights of 100-130 kDa, 55 kDa and 26-33 kDa on the SDS-PAGE gel (Figure. 1). The band with a molecular weight of 100-130 kDa was the most strongly reacting single band as detected by Western blotting with the HRP labeled mucin.

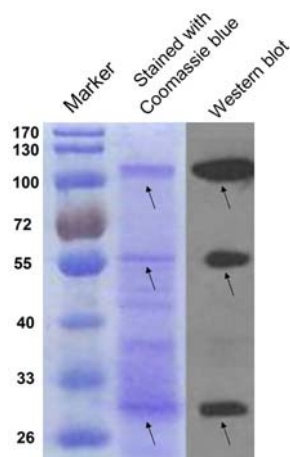
Assumption of the potential target proteins by LC-MS/MS and data analysis. The three bands of 100-130 kDa, 55 kDa and 26-33 kDa from *L. plantarum* CGMCC 1258 were all excised from the gel and subjected to LC-MS/MS, followed by analysis using SEQUEST, Trans-proteomics Pipeline and FASTA, as described in the Methods ProteinProphet algorithm (27) (Figures 2A-2C). Four proteins, pyruvate oxidase (PO), cell division initiation protein FtsQ (CD), GTP-binding protein TypA (GBA), and IMP were predicted to be the putative target protein (Table 3), as the coincidence rate was more than 90% for each to the *L. plantarum* WCFS1 protein pool.

#### 4.1.2. Cloning, expression and purification of the potential target proteins in *E. coli*

The target genes encoding the putative target proteins (i.e GBA, PO, CD and IMP) were amplified by polymerase chain reaction (PCR) from DNA of *L. plantarum* CGMCC1258. After electrophoresis, the PCR products revealed several bands ranging from 500 bp to over 1500 bp as detected on 3% agarose gel (Figure. 3A). The bands were extracted and ligated into the PET32B vector. The resultant plasmids were transfected into competent *E. coli* DH10B. DNA sequencing verified that the amplified genes from *L. plantarum* CGMCC1258 were the same as those of the *L. plantarum* WSFC1 when compared to GenBank entries.

Then, the plasmids containing the desired genes were transfected into *E. coli* BL21 (DE3). After 3-h incubation with IPTG, the cells were sonicated, and the target proteins were extracted and purified using His-tag purification beads, as described in the Methods section. The eluted fractions were separated on 10% SDS-PAGE, and the protein adhering to the gastric mucin was detected by Western blotting using HRP-labeled mucin for blotting (Figures. 3B and 3C). Target proteins accounted for more than 90% of the total proteins prepared from the bacteria, with a concentration of 500  $\mu$ g/mL. However, only IMP-2 exhibited a strong mucin-binding reaction, generating a band at 20-30 kDa by Western blotting, which indicated that IMP-2 was the adhesive protein. No positive band was found in any other transfected cell lysates (Figure. 3C).

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**Figure 1.** Identification of the extracted surface layer protein responsible for adhesion, using HRP-mucin for blotting. Left two lanes show molecular marker (kDa) and the extracted surface layer protein (arrow) stained with Coomassie brilliant blue R-250 in SDS-PAGE analysis; the right lane shows the extracted surface layer protein (arrow) in Western blot analysis.

### 4.2. Identification of MIMP

#### 4.2.1. Primary amino acid sequence analysis of IMP-2

The amino acid sequence encoding the mature IMP-2 was subjected to a number of analyses. IMP455-755 was searched using BLASTP with default parameters against NCBI non-redundant database. A conserved domain, which was described as PF09586 in a bacterial membrane protein Yfh0, was found in the 1-217 (IMP455-672) region. By searching the NCBI Conserved Domains Database, 1710 proteins (six proteins with score>200) were found to be similar to PF09586. The gi|254557086 protein of *L. plantarum* JDM1 and the gi|227896284 protein of *L. plantarum* subsp. ATCC 14917 were selected after searching GenBank by annotation keywords with limitation in *L. plantarum*. In a homology search in the UniProt database by using BLASTP, four new proteins with similar structure to IMP, B2G7B3, B3XQC4, B2GCS0, and A9UJX3, were identified. Regions corresponding to the Yfh0 domain within these sequences were extracted, respectively, according to the alignment of PFAM family (<http://pfam.sanger.ac.uk/>). Predicted isoelectric points (PI) of these proteins are shown in Table 6. Multiple Sequence Alignment analyses indicated that IMP510-688 (55-133) may include the domain of interest (Figure 4A). The hydrophobicity of IMP455-755 was calculated and is shown in Figure 4B. There was a similar distribution of hydrophilic and hydrophobic amino acid residues, with the hydrophobic and hydrophilic residues of the mature protein evenly alternating as exemplified by the hydrophobicity plot.

#### 4.2.2. Cloning and expression of gene sequences encoding the mature or truncated forms of IMP-2 and purification of the recombinant proteins

PCR products encoding the mature IMP-2 and the six N- or C-terminal IMP-2 truncated forms were

successfully cloned in *E. coli* DH10B and expressed in *E. coli* BL21 (DE3) (Figure 5A).

#### 4.2.3. Identification of the ability of recombinant IMP forms to adhere to the gastric mucin by SDS-PAGE and Western blotting with enhanced chemiluminescence (ECL).

On SDS-PAGE gels stained with Coomassie blue, each of the recombinant proteins was shown as a single distinct band, with a molecular weight corresponding to the calculated value (Figure 5). As a preliminary test for the adhesive property of the recombinant truncated IMP forms, proteins separated by SDS-PAGE were examined by western blotting with ECL (Figure 5 C). The C-terminal truncated forms, IMP455-575, IMP455-635, and IMP455-695 and the N-terminal truncated form, IMP515-755, adhered to the gastric mucin and chemiluminesced efficiently, while the N-terminal truncated forms, IMP635-755 and IMP575-755 exhibited no adhesion ability. In addition, Western blotting showed adhesion only for the residues IMP515-575 (Figures 5 A&C). Since the truncated proteins containing residues IMP515-575 from IMP-2 exhibited the ability to adhere to the gastric mucin, and the ability was absent in those forms without these residues, IMP515-575 was determined to be the smallest domain essential for the ability of IMP to adhere to the gastric mucin, and defined as the micro integral membrane protein (MIMP). Also, this domain was encompassed within the larger IMP510-688 indicated by the Multiple Sequence Alignment analyses.

#### 4.2.4. Recognition of polyclonal anti-MIMP antibodies with the recombinant MIMP

The specificity of the polyclonal anti-MIMP antibodies was determined by Western blotting and ELISA. Western blotting showed that the polyclonal antibodies obtained from immunizing the New Zealand rabbit with recombinant MIMP specifically reacted with the purified MIMP from *E. coli*, even when the concentration of MIMP was as low as 1 pg (Figure 6). ELISA results also showed that the polyclonal antibodies reacted to MIMP (1 mg/L) (Table 4).

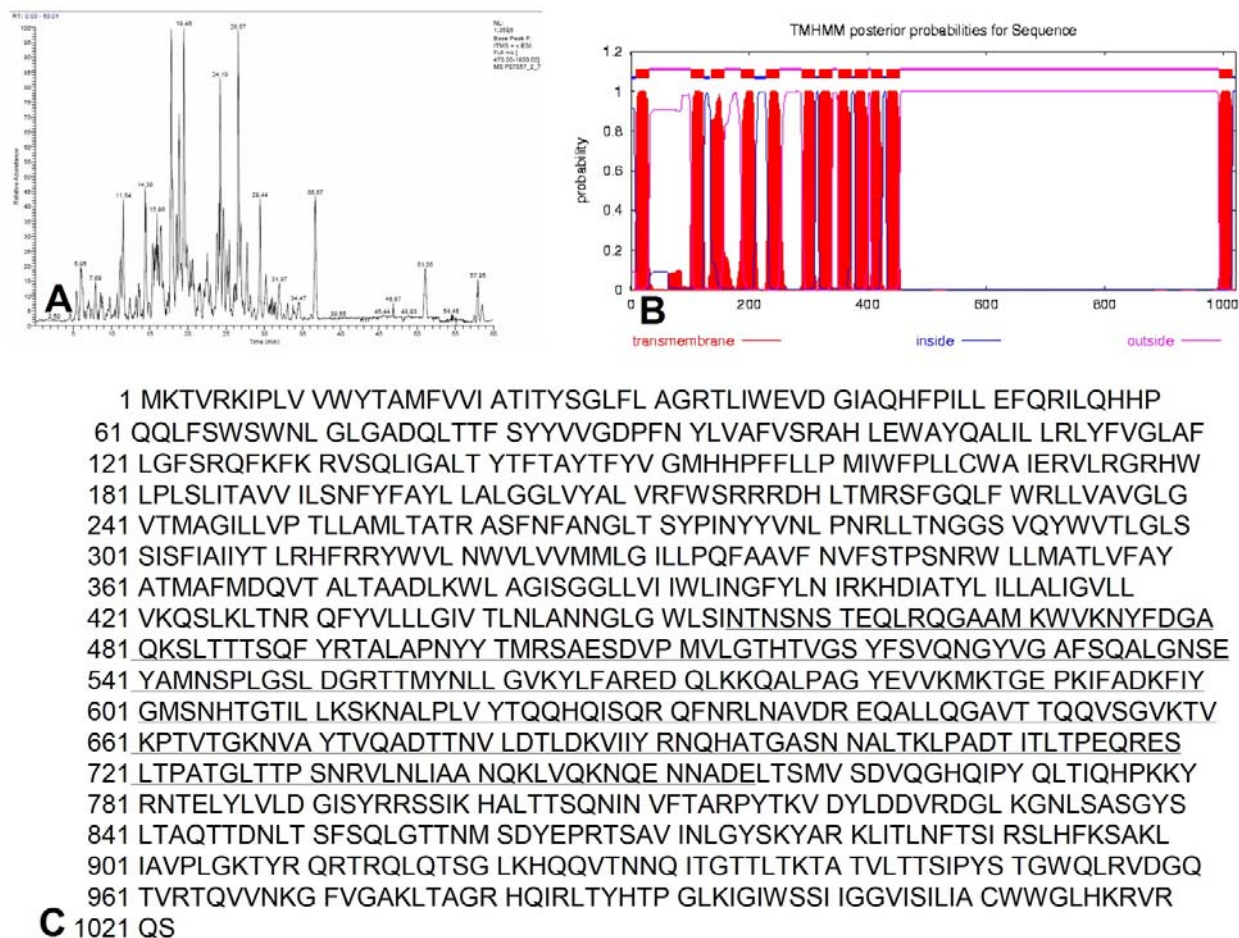
#### 4.2.5. Detection of IMP isolated and purified from *L. plantarum* with anti-MIMP polyclonal antibodies

IMP was detected by polyclonal anti-MIMP antibodies, and the positive band corresponded to 100-130 kD (Figure 7).

### 4.3. MIMP competitively adheres with EIEC/EPEC to NCM460 cells

Adhesion rates of EIEC ATCC43893 (O124:NM) to NCM460 cells ranged between 15% and 28%, while adhesive rates of EPEC ATCC43887 (O111:NM) to NCM460 cells ranged between 20% and 45% (Figure 8). The adhesion rates decreased significantly following co-culture with *L. plantarum* or purified MIMP, compared with those in the EIEC/EPEC group (all  $P < 0.001$ ). However, the inhibitory effect of MIMP was inhibited by pre-incubation with the anti-MIMP polyclonal antibodies to MIMP (all  $P < 0.001$ , Figures. 8A&B).





**Figure 2.** Maps of LC-MS/MS for the 30-40 kDa protein and Thermo Fisher SEQUEST Microsoft data analysis and identification. (A), Expression profiles of the proteins. (B), Posterior label probability plot to predict which regions are likely to be localized intracellularly, extracellularly, or membrane-bound. (C), Amino acid sequence of IMP-2.

#### 4.4. MIMP adheres to and matures iDCs as detected by flow cytometry

*L. plantarum* and MIMP modulated the iDC phenotype by up-regulating MHC-II and activating the expression of the costimulatory molecules CD80 and CD86 at all examined ratios, from 10 to 1,000 cfu of *L. plantarum* per iDC and 0.01 µg/ml to 1 µg/ml of MIMP (Figure 9).

## 5. DISCUSSION

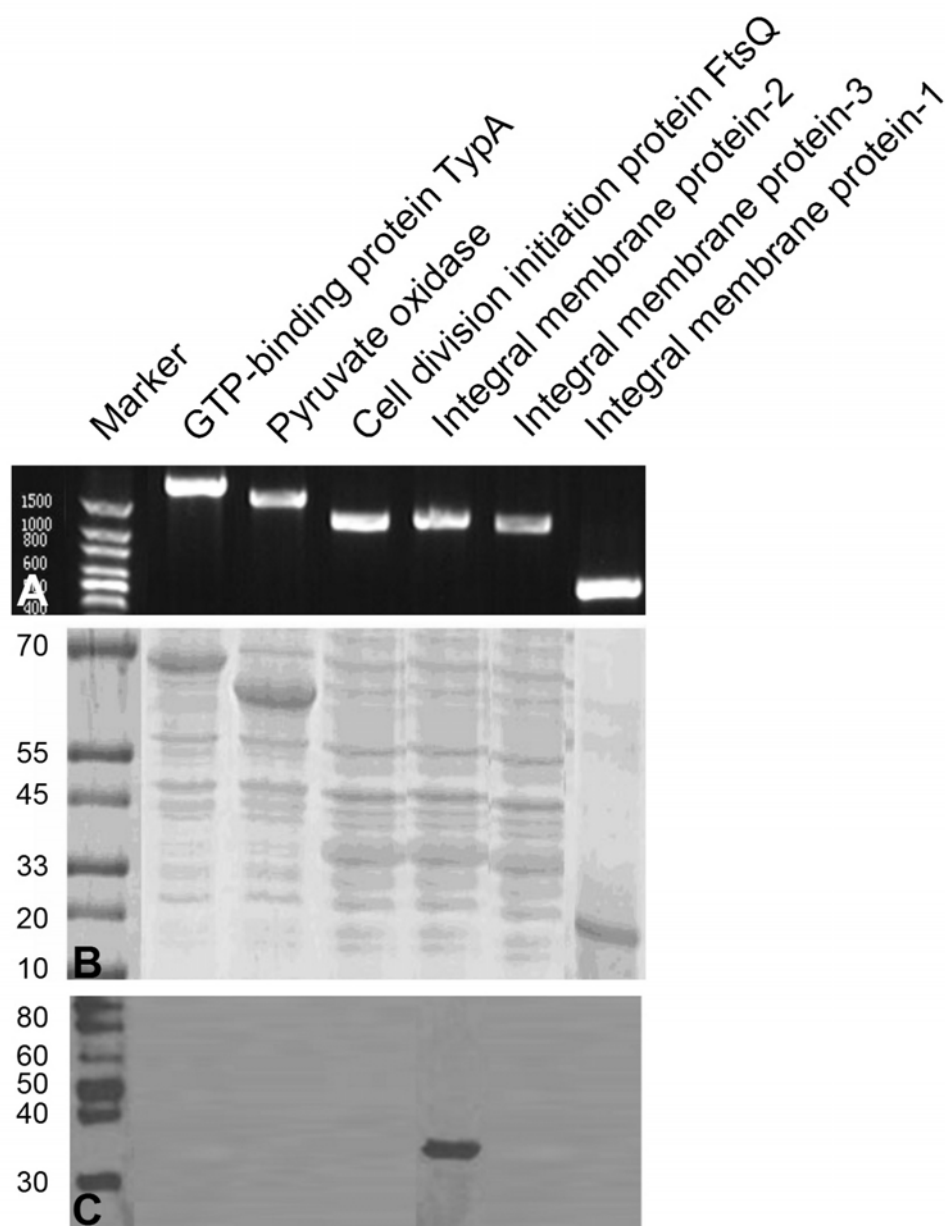
In the study, we first identified IMP-2 as the SLP peptide of *L. plantarum* CGMCC 1258 that was responsible for adhesion to gastric mucin. Moreover, we characterized MIMP (i.e. IMP515-575) as the smallest domain within IMP-2 that possesses the ability to adhere to the gastric mucin and competitively adhere to intestinal epithelial cells. We believe that these findings are significant to both biotechnical and clinical fields.

In the present study, we first applied molecular techniques, including SDS-PAGE, Western blotting and cloning to identify IMP-2 as the protein responsible for the

adhesion ability of *L. plantarum*. These findings demonstrated that the combination of modern bioinformatics and established molecular techniques is a powerful tool for the identification and purification of targeted proteins with specific biological functions.

In the present study, MIMP was first generated in and purified from *E. coli* by using genetic engineering techniques. IMP was, then, able to be purified directly from the *L. plantarum* strain, which was recognized by the polyclonal anti-MIMP antibodies, and could be used to produce MIMP by an appropriate prolease enzyme. These findings demonstrated that MIMP can be produced by the two approaches as describe above. In addition, the present study revealed the antigenicity of MIMP, at least, in rabbits.

In the present study, we applied a novel approach based on modern bioinformatics and biotechniques to identify the particular SLP of *L. plantarum* responsible for its adhesion property and the exact functional domain of the protein. First, we used LC-MS/MS to detect a pool of

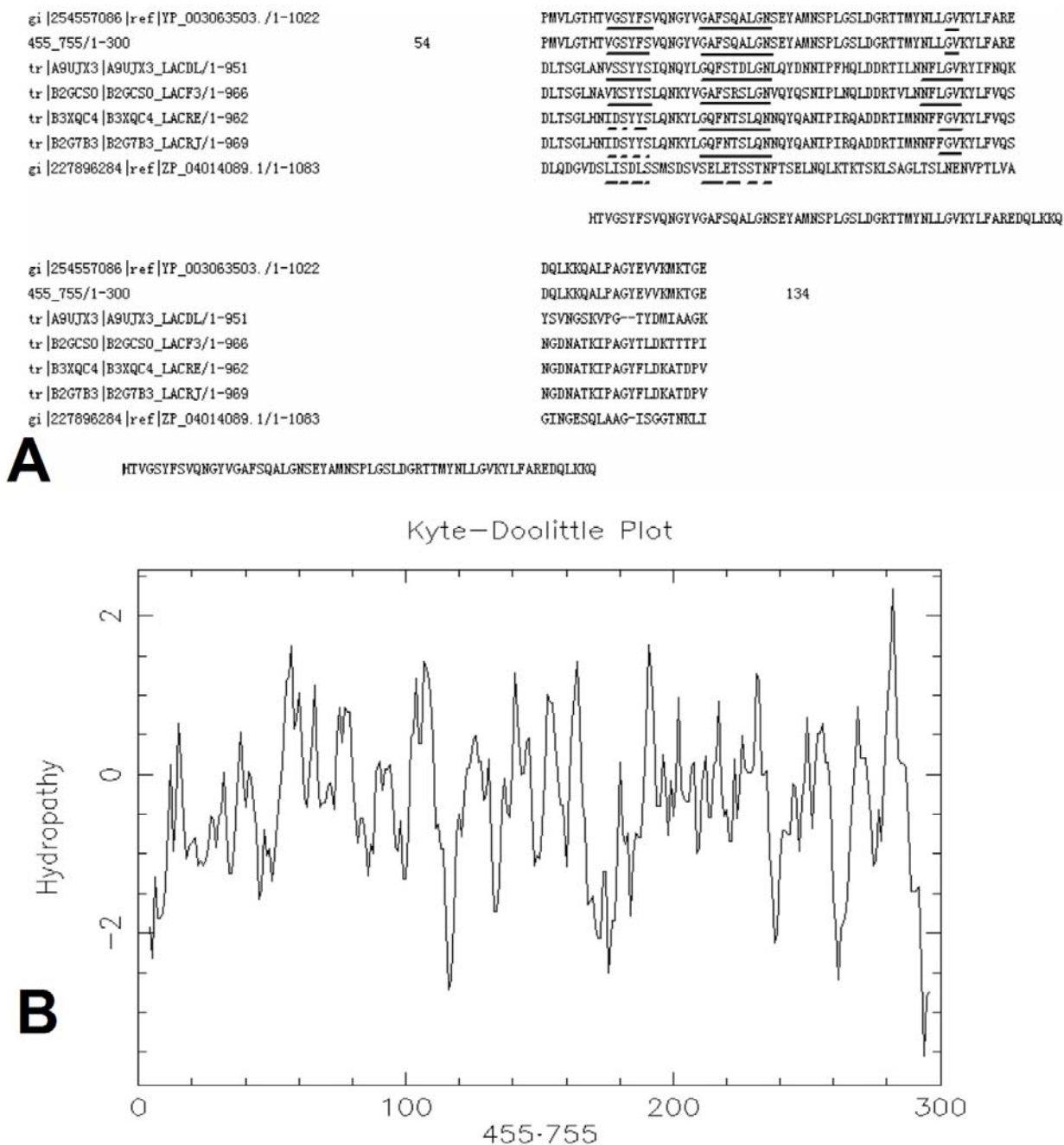


**Figure 3.** Expression and identification of the proteins. (A). PCR amplification of the genes from *L. plantarum* CGMCC No1258. (B), SDS-PAGE analysis of the expressed recombinant proteins in *E. coli*. (C), Western blot analysis of the recombinant proteins using HRP-mucin for blotting.

possible *L. plantarum* SLPs adhering to gastric mucin, which narrowed the number of possible adhesive proteins from 83 to 5; IMP (the only membrane protein) was indicated as the adhesive protein among the candidate population. Western blotting verified that IMP-2, the medium section of IMP, exhibited a distinct and strong reaction with the gastric mucin, generating a band at 20-30 kDa. Primary amino acid sequence analysis of IMP-2 with BLASTP was performed to predict the smallest domain responsible for its adhesion property. IMP455-672 was predicted by BLASTP as the possible domain for adhesion. Accordingly, gene sequences encoding the mature or

truncated forms of IMP-2 were successfully cloned and expressed. IMP515-575, i.e. MIMP, was identified as the active domain. Our experience clearly demonstrates that bioinformatics plays at least two important roles in hypothesis-driven research: one is to predict the interesting protein and the functional domain for further experimental identification, while the other is to verify our experimental data in turn. For the domain detection, we used the bioinformatics approach to analyze the primary amino acid sequence; however, we found the predicted domain IMP510-688 was too large. Therefore, we further constructed six N- or C-terminal IMP-2 truncated forms to

## Molecular identification of MIMP



**Figure 4.** Multiple Sequence Alignment and hydrophobicity analyses of IMP455-755. (A), Similarity of the Yfh0 regions of *L. plantarum* IMP to other proteins identified in Conserved Domains Database (Score>200) as performed by ClustalW. Broken underline indicates potential motifs for the smallest functional domain in IMP at different locations: IDSYYS of B3XQC4 and B2G7B3 and LISDLS of gi|227896284 correspond to VGSYFS of the consensus sequence, and SELETSSNT of gi|227896284 corresponds to GAFSQALGN of the consensus sequence. (B), Hydrophobicity of IMP455-755 is calculated according to Kyte and Doolittle with a window of seven amino acids, which shows a similar distribution of hydrophilic and hydrophobic amino acid residues along the mature proteins with evenly alternating hydrophobic and hydrophilic residues, as exemplified by the hydropathy plot.

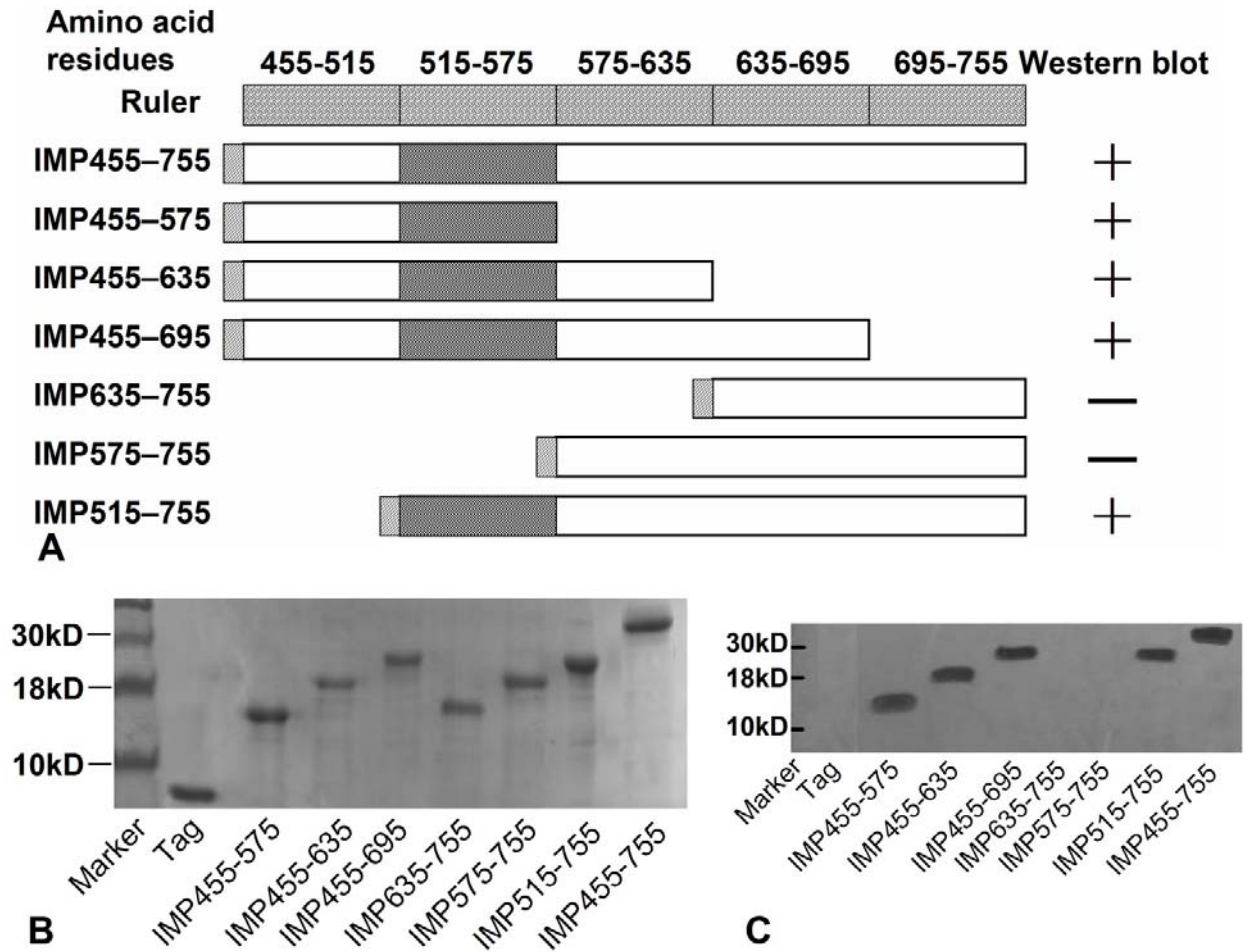
specifically identify the domain; using a comparison of gels stained with Coomassie brilliant blue and banding patterns detected on PVDF membrane Western blots, the domain protein MIMP was identified.

In the process of protein cloning and expression, we used *pET16b* to add cutting sites for the *Bgl*II and *Xho*I restriction endonucleases near multiple cloning sites as the cross-reference. We attained protein expression up to 90%

Table 6. Predicted isoelectric points of the Yfh0-related proteins

Yfh0-related proteins	Predicted isoelectric points
IMP455-755(2-297)	9.57
gji254557086(16-999)	9.96
gji227896284(10-1050)	9.03
B2G7B3(27-947)	9.49
B3XQC4(20-940)	9.47
B2GCS0(20-943)	9.64
A9UJX3(20-922)	9.71

Molecular identification of the domain within the integral membrane protein of *Lactobacillus plantarum* responsible for adhering to gastric mucin

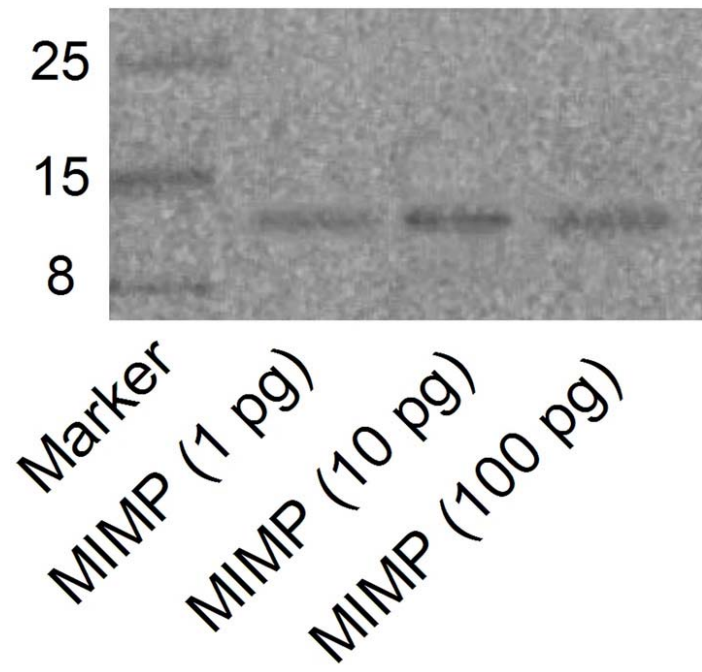


**Figure 5.** Adhesive properties of the mature and truncated IMP-2 forms. (A) Schematic presentation of the recombinant mature and truncated IMP-2 forms and their adhesive properties as verified by Western blotting using HRP-mucin. Tags in the left of the protein section consists of 17 amino acids; The domain protein MIMP is shown in shadows; Non-adhesive IMP forms is shown as blank; Ruler is in the above of the figure 5A. (+) indicates that the mature and truncated IMP-2 forms are able to adhere to mucin; and (-) indicates that the truncated IMP-2 forms do not have adhesive ability. (B), Recombinant proteins of the mature and truncated forms as detected by SDS-PAGE gel stained with Coomassie blue. (C), Western blot showed the adhesion properties of some truncated IMP forms.

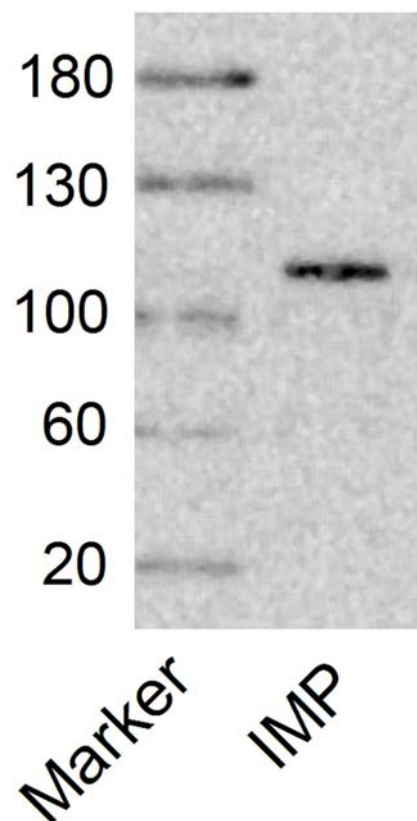
(500 µg/ml). Furthermore, we chose to add a tag of 198 amino acids (Table 1) for cloning to enhance the expression level of truncated proteins (less than 200 amino acids). The protein expression of up to 500 µg/ml was achieved in both situations with this strategy.

*L. plantarum* exhibits its therapeutic effects, mainly, by preventing and ameliorating the pathogen-

induced disruption of the TJ structure and redistribution of TJ proteins (31-32). In the present study, we further observed that MIMP, like *L. plantarum*, was able to antagonize the adhesion of EIEC and EPEC as they adhere to NCM460 colonic cells. Thus, we postulated that *L. plantarum* may exert its protective effects through MIMP. In other words, MIMP may possess most (if not all) therapeutic activities of *L. plantarum* CGMCC1258,

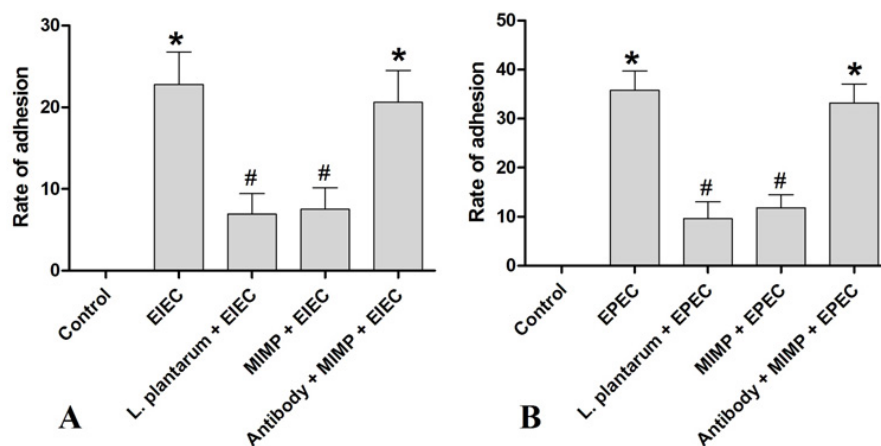


**Figure 6.** Sensitivity of polyclonal anti-IMP2 antibodies in recognizing MIMP as detected by Western blotting.

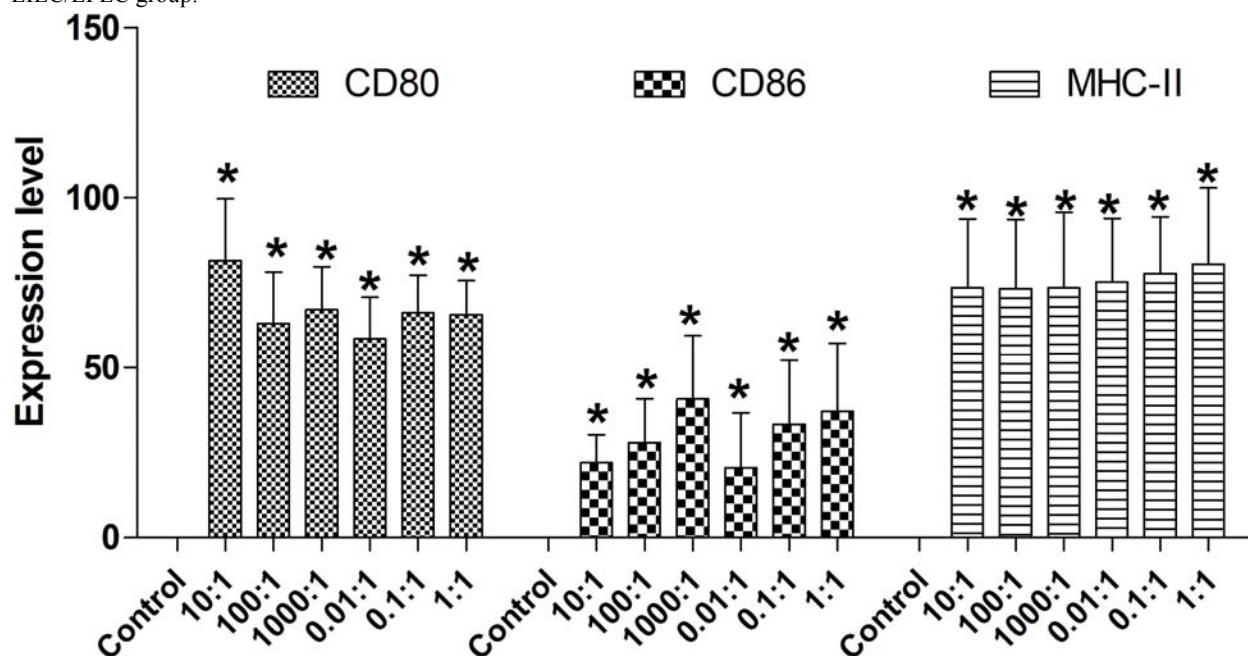


**Figure 7.** Verification of IMP-2 ability to adhere to mucin as detected by Western blotting.





**Figure 8.** Competing adhesion analysis showing that MIMP reduces the adhesion rates of enteroinvasive *E. coli* (A) or enteropathogenic *E. coli* (B) to NCM460 cells; the inhibitory effect was nearly abolished by the addition of polyclonal anti-MIMP antibodies. The data are expressed as mean value  $\pm$  standard deviation (SD) of three separate experiments. \*  $P < 0.001$ , compared with the control group; #  $P < 0.001$ , compared with EIEC/EPEC or polyclonal anti-MIMP antibodies + MIMP + EIEC/EPEC group.



**Figure 9.** *L. plantarum* and MIMP stimulates maturation of immature dendritic cells (iDCs) as detected by flow cytometry. *L. plantarum* and MIMP modulate the iDC phenotype by up-regulating MHC-II and activating the expression of the co-stimulatory molecules CD80 and CD86 in a concentration-dependent manner.

including maintaining intestinal epithelial barrier function and resolving intestinal inflammation by attenuating pathogen-induced decreased TER, improving the altered TJ structure, inhibiting the increased intestinal permeability, and decreasing the production of pro-inflammatory cytokines. In fact, we have investigated these activities of MIMP in a parallel study functionally characterizing MIMP.

It was noted that, like other SLPs (15, 33-35), the antagonizing effect of MIMP on the adhesion of

EIEC/EPEC to NCM460 cells was suppressed by the pre-treatment of polyclonal antibodies against MIMP prepared in the rabbit. Thus, one may raise some critical concerns, i.e. whether the anti-MIMP antibodies are also produced in humans, whether MIMP also produces systemic effects and, if both answers are yes, whether the anti-MIMP antibodies would compromise the systemic effects of MIMP in humans. However, we believe that this is unlikely because MIMP exhibits its therapeutic effects mainly through local adhesion and interaction with the intestinal epithelial cells, and subsequent regulation in the intestinal

permeability, immune response and inflammation. In addition, even if MIMP were translocated into the blood, anti-MIMP antibodies would neutralize it and thus minimize any potential adverse effects. Further studies are required to clarify this issue.

The findings obtained from the present study outline a very important approach, by identifying the adhesion domain in SLP of *L. plantarum*, to further broadening and extending our current understanding of *L. plantarum* and its therapeutic effects on the cellular and molecular mechanisms of intestinal barrier dysfunction and intestinal inflammation. Taken together with our functional study, our findings may lead to changes in the pharmacological application of *L. plantarum* and, most importantly, to the understanding of some intestinal diseases, such as inflammatory bowel disease. Nevertheless, further studies are required to confirm the mechanisms of action of MIMP and its short- and long-term clinical efficacy and safety.

In conclusion, we identified MIMP, i.e. IMP515-575, as the adhesion domain of the SLP of *L. plantarum*. The present study provides a foundation for our parallel study that determines its functions, along with *L. plantarum* CGMCC1258, as well as any future studies on MIMP focused on its mechanisms of action, clinical efficacy and safety.

## 6. ACKNOWLEDGMENT

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## 7. REFERENCES

1. F. Backhed, R. E. Ley, J. L. Sonnenburg, D. A. Peterson and J. I. Gordon: Host-bacterial mutualism in the human intestine. *Science*, 307(5717), 1915-20 (2005)
2. S. Nuding, K. Fellermann, J. Wehkamp and E. F. Stange: Reduced mucosal antimicrobial activity in Crohn's disease of the colon. *Gut*, 56(9), 1240-7 (2007)
3. J. M. Rhodes: The role of *Escherichia coli* in inflammatory bowel disease. *Gut*, 56(5), 610-2 (2007)
4. Z. H. Liu, Y. L. Ma, T. Y. Shen, H. Q. Chen, Y. K. Zhou, P. Zhang, M. Zhang, Z. X. Chu and H. L. Qin: Identification of DC-SIGN as the receptor during the interaction of *Lactobacillus plantarum* CGMCC 1258 and dendritic cells. *World J Microbiol Biotechnol.* (2010)
5. C. Fiocchi: One commensal bacterial molecule--all we

need for health? *N Engl J Med*, 353(19), 2078-80 (2005)

6. J. C. Alverdy, R. S. Laughlin and L. Wu: Influence of the critically ill state on host-pathogen interactions within the intestine: gut-derived sepsis redefined. *Crit Care Med*, 31(2), 598-607 (2003)  
doi:10.1097/01.CCM.0000045576.55937.67
7. R. B. Sartor: Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology*, 126(6), 1620-33 (2004)
8. J. Ewaschuk, R. Endersby, D. Thiel, H. Diaz, J. Backer, M. Ma, T. Churchill and K. Madsen: Probiotic bacteria prevent hepatic damage and maintain colonic barrier function in a mouse model of sepsis. *Hepatology*, 46(3), 841-50 (2007)
9. T. Matsuzaki, A. Takagi, H. Ikemura, T. Matsuguchi and T. Yokokura: Intestinal microflora: probiotics and autoimmunity. *J Nutr*, 137(3 Suppl 2), 798S-802S (2007)
10. Z. Liu, P. Zhang, Y. Ma, H. Chen, Y. Zhou, M. Zhang, Z. Chu and H. Qin: *Lactobacillus plantarum* prevents the development of colitis in IL-10-deficient mouse by reducing the intestinal permeability. *Mol Biol Rep.* (2010)
11. A. Garcia-Lafuente, M. Antolin, F. Guarner, E. Crespo and J. R. Malagelada: Modulation of colonic barrier function by the composition of the commensal flora in the rat. *Gut*, 48(4), 503-7 (2001)
12. M. G. Gareau, J. Jury, G. MacQueen, P. M. Sherman and M. H. Perdue: Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. *Gut*, 56(11), 1522-8 (2007)
13. B. L. Buck, E. Altermann, T. Svingerud and T. R. Klaenhammer: Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol*, 71(12), 8344-51 (2005)
14. G. Francius, D. Alsteens, V. Dupres, S. Lebeer, S. De Keersmaecker, J. Vanderleyden, H. J. Gruber and Y. F. Dufrene: Stretching polysaccharides on live cells using single molecule force spectroscopy. *Nat Protoc*, 4(6), 939-46 (2009)
15. K. C. Johnson-Henry, K. E. Hagen, M. Gordonpour, T. A. Tompkins and P. M. Sherman: Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. *Cell Microbiol*, 9(2), 356-67 (2007)
16. S. Avall-Jaaskelainen, U. Hynonen, N. Ilk, D. Pum, U. B. Sleytr and A. Palva: Identification and characterization of domains responsible for self-assembly and cell wall binding of the surface layer protein of *Lactobacillus brevis* ATCC 8287. *BMC Microbiol*, 8, 165 (2008)
17. M. Sara and U. B. Sleytr: S-Layer proteins. *J Bacteriol*,

182(4), 859-68 (2000)

18. M. F. Bernet, D. Brassart, J. R. Neeser and A. L. Servin: Lactobacillus acidophilus LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut*, 35(4), 483-9 (1994)

19. B. Wang, H. Wei, J. Yuan, Q. Li, Y. Li, N. Li and J. Li: Identification of a surface protein from Lactobacillus reuteri JCM1081 that adheres to porcine gastric mucin and human enterocyte-like HT-29 cells. *Curr Microbiol*, 57(1), 33-8 (2008)

20. J. M. Wells and A. Mercenier: Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. *Nat Rev Microbiol*, 6(5), 349-62 (2008)

21. H. Qin, Z. Zhang, X. Hang and Y. Jiang: L. plantarum prevents enteroinvasive Escherichia coli-induced tight junction proteins changes in intestinal epithelial cells. *BMC Microbiol*, 9, 63 (2009)

22. M. Zhang, X. Q. Wang, Y. K. Zhou, Y. L. Ma, T. Y. Shen, H. Q. Chen, Z. X. Chu and H. L. Qin: Effects of oral Lactobacillus plantarum on hepatocyte tight junction structure and function in rats with obstructive jaundice. *Mol Biol Rep* (2009)

23. Z. X. Chu, H. Q. Chen, Y. L. Ma, Y. K. Zhou, M. Zhang, P. Zhang and H. L. Qin: Lactobacillus plantarum Prevents the Upregulation of Adhesion Molecule Expression in an Experimental Colitis Model. *Dig Dis Sci* (2009)

24. H. L. Qin, J. J. Zheng, D. N. Tong, W. X. Chen, X. B. Fan, X. M. Hang and Y. Q. Jiang: Effect of Lactobacillus plantarum enteral feeding on the gut permeability and septic complications in the patients with acute pancreatitis. *Eur J Clin Nutr*, 62(7), 923-30 (2008)

25. M. Rojas, F. Ascencio and P. L. Conway: Purification and characterization of a surface protein from Lactobacillus fermentum 104R that binds to porcine small intestinal mucus and gastric mucin. *Appl Environ Microbiol*, 68(5), 2330-6 (2002)

26. V. Kertesz, H. M. Connelly, B. K. Erickson and R. L. Hettich: PTMSearchPlus: software tool for automated protein identification and post-translational modification characterization by integrating accurate intact protein mass and bottom-up mass spectrometric data searches. *Anal Chem*, 81(20), 8387-95 (2009)

27. A. I. Nesvizhskii, A. Keller, E. Kolker and R. Aebersold: A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem*, 75(17), 4646-58 (2003)

28. G. D. Pullinger, R. Sowdhamini and A. J. Lax: Localization of functional domains of the mitogenic toxin of Pasteurella multocida. *Infect Immun*, 69(12), 7839-50

(2001)

29. D. Moncada, K. Keller and K. Chadee: Entamoeba histolytica cysteine proteinases disrupt the polymeric structure of colonic mucin and alter its protective function. *Infect Immun*, 71(2), 838-44 (2003)

30. F. Sallusto and A. Lanzavecchia: Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med*, 179(4), 1109-18 (1994)

31. B. Wang, J. Li, Q. Li, H. Zhang and N. Li: Isolation of adhesive strains and evaluation of the colonization and immune response by Lactobacillus plantarum L2 in the rat gastrointestinal tract. *Int J Food Microbiol*, 132(1), 59-66 (2009)

32. M. Botes, B. Loos, C. A. van Reenen and L. M. Dicks: Adhesion of the probiotic strains Enterococcus mundtii ST4SA and Lactobacillus plantarum 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments. *Arch Microbiol*, 190(5), 573-84 (2008)

33. X. Chen, J. Xu, J. Shuai, J. Chen, Z. Zhang and W. Fang: The S-layer proteins of Lactobacillus crispatus strain ZJ001 is responsible for competitive exclusion against Escherichia coli O157:H7 and Salmonella typhimurium. *Int J Food Microbiol*, 115(3), 307-12 (2007)

34. H. G. Kim, N. R. Kim, M. G. Gim, J. M. Lee, S. Y. Lee, M. Y. Ko, J. Y. Kim, S. H. Han and D. K. Chung: Lipoteichoic acid isolated from Lactobacillus plantarum inhibits lipopolysaccharide-induced TNF-alpha production in THP-1 cells and endotoxin shock in mice. *J Immunol*, 180(4), 2553-61 (2008)

35. S. A. van Hijum, S. Kralj, L. K. Ozimek, L. Dijkhuizen and I. G. van Geel-Schutten: Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol Mol Biol Rev*, 70(1), 157-76 (2006)

**Abbreviations:** MIMP: micro integral membrane protein; TJ tight junction; EPEC enteropathogenic *Escherichia coli*; EIEC enteroinvasive *E. Coli*; IMP integral membrane protein; SLP surface layer proteins

**Key Words:** Molecular Identification, Protein Domain, Surface Layer Protein, *Lactobacillus plantarum*, NCM460

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