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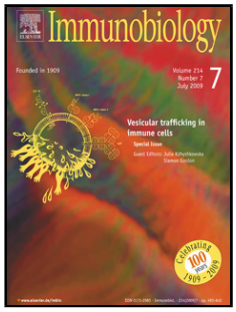

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Molecular identification of potential Th1/Th2 responses-modulating bacterial genes using suppression subtractive DNA hybridization

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Abbreviations: CFU, colony-forming units; EPS, exopolysaccharide; GTF, glucosyltransferase; LTA, lipoteichoic acid; MAMPs, microbe-associated molecular patterns; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; OD,optical density, PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGN, peptidoglycan; PPT, pyruvyltransferase; SEA, staphylococcal enterotoxin A; Th1, T Helper Cell Type 1; Th2, T Helper Cell Type 2; WTA, wall teichoic acid

Key words: Cytokine; *Lactobacillus fermentum*; optical density; PBMCs; probiotics; suppression subtractive hybridization

Abstract

Background and Objectives: We characterized by co-incubation with peripheral blood mononuclear cells (PBMC) the immunomodulating potential of a number of lactobacilli isolated from an African fermented food. Two strains with different immune modulating properties were genetically compared by Suppression Subtractive Hybridisation (SSH).

Methods: From 48 *Lactobacillus* strains isolated from *Kimere, African* fermented pearl millet dough, 10 were selected based on their bile salt tolerance. Their effects on secretion by PBMCs of the T-helper cells Th1- and Th2-cytokines IFN-! and IL-4, respectively, in the presence or absence of staphylococcal enterotoxin A-(SEA-) were assessed. To study the genetic basis of different immune-modulating properties, a subtracted cDNA library for *L. fermentum* strains K1-Lb1 (Th1 inducer) and K8-Lb1 (Th1 and Th2 suppressor) was constructed using SSH. Finally, adhesion of these strains to hydrocarbons (relative hydrophobicity) and to human HT-29 colonic epithelial cell line was assessed.

Results: Two strains, K1-Lb1 and K4-Lb6, induced basal IFN-! secretion. Four strains, K1-Lb6, K6-Lb2, K7-Lb1, and K8-Lb1 diminished INF-! secretion by SEA-stimulated PBMCs. All strains, except K1-Lb1, K2-Lb4, and K9-Lb3, inhibited SEA-stimulated IL-4 secretion. Comparing the genomes of K1-Lb1 and K8-Lb1 by SSH indicated that K1-Lb1 is able to synthetize polysaccharides, for the synthesis of which K1-Lb8 appears to lack enzymes. A difference in the hydrophobicity properties of the surfaces of both strains indicated that this has impact on their surface.

Conclusion: The K1-Lb1-specific sequences encoding putative glycosyltransferases and enzymes for polysaccharides synthesis may account for the observed differences in immunomodulation and surface properties between the two strains and for mediating potential probiotic effects.

1 Introduction

Aberrant gut microbiota and allergic and other inflammatory disorders can shift the Th1/Th2 cytokine balance towards a Th2 response, leading to activation of Th2 cytokines and the release of interleukin-4 (IL-4), IL-5, and IL-13 as well as IgE production (Michail 2009). Accordingly, oral intake of probiotics is suggested to prevent or alleviate allergic and other inflammatory disease, specifically those related to inappropriate immune functions associated with Th1/Th2 immune responses (Kuitunen, 2009 and Lee and Bak, 2011). Probiotic bacteria and their components have been shown to modulate Th1/Th2 immune response(s) of antigen/allergen-stimulated immune cells (e.g., PBMCs) both *in vitro* and *in vivo* (Foligne et al., 2007; Forsythe et al., 2007; Ghadimi et al., 2008; Helwig et al., 2006; Pochard et al., 2002). Probiotic bacteria can enhance IFN-production and decrease IgE and antigen-induced TNF! , IL-5, and IL-10 secretion (Michail, 2009). They and their components, respectively, can potentially modulate the Toll-like receptors and ameliorate inflammatory status (Foligne et al., 2007; Winkler et al., 2007; Zakostelska et al., 2011).

Selection of strains for application as probiotics focuses on two main properties: i) adaptability to the gastrointestinal environment and ii) health promoting or functional properties. These selection schemes include survival at low pH and in the presence of bile salts, adhesion to intestinal epithelial cells, colonization of the gut, maintenance of microbial balance, non-pathogenicity to the host, resistance to technological challenges such as processing and distribution, and, last but not least, ability to confer health benefits to the host (FAO/WHO 2002; Heller, 2001; Schrezenmeir and de Vrese, 2001). It should be noted that not every single probiotic strain needs to possess all these characteristics. According to recent scientific evidence, bacteria need not necessarily be 'live' to exert immunomodulation effects as both live and dead cells as well as bacterial DNA were shown to exert some potential health benefits (Ghadimi et al., 2008; Ghadimi et al., 2011; Laudanno et al., 2006; Winkler et al., 2007). Depending on functional targets like colonization of the gut, exclusion of putrefactive bacteria or

pathogens, and modulation of microbial metabolism, survival in the gut, however, may be essential in order to ensure that probiotics reach the intended site in an active state (Schrezenmeir and de Vrese, 2001; Salminen and Isolauri, 2006).

As the functional properties of probiotic bacteria are known to be strain-specific, selection and assessment of potential probiotic isolates is important for development of new efficient probiotic preparations (Larson et al., 2009). With this respect, previous comparative *in vitro* and *in vivo* studies on the effects of probiotic strains on immune function of a range of immune cells have shown significant differences in the cytokine profiling of the *Lactobacillus acidophilus* strains. For example, Holvoet et al., (2013) determined the effects of probiotics on Th2-skewed cells, classified probiotic strains with anti-allergic potential and showed that cytokine profiles induced by probiotics were strain specific. Dong et al., (2012), Drago et al., (2010), Pérez-Cano et al., (2010), Snel et al., (2011) and Vissers et al., (2010 and 2011) compared the immunomodulatory effects of different probiotic strains and showed that modulation of expression of some cytokines like IFN-! and IL-4 was strongly strain-specific. Donkor et al., (2012) showed that although all tested strains of probiotic bacteria had the capacity to induce pro- and anti-inflammatory cytokine production by cell lines and PBMCs, the magnitude of production of each cytokine varied depending on the strain. This strain-specific modulation of expression of cytokines has been attributed to differences in bacterial genomes (van Hemert et al., 2010 and Meijerink et al., 2010).

Regarding probiotic characteristics like acid and bile tolerance, antibiotic susceptibility, antimicrobial activity, cell adhesion and antioxidant activity, Dixit et al. (2013), Jamaly et al. (2011), Larsen et al. (2009), Lewandowska et al. (2005) and Venkatesan et al., (2012) have shown significant differences in such characteristics when comparing different strains.

In Africa, several fermented cereal products are produced and consumed. Communities around Mount Kenya have fermented cereal gruel as their daily fermented food. *Kimere* is a traditional fermented pearl millet (*Pennisetum glaucum*) gruel, which is consumed among the Mbeere community. This diet plays a major role in nutrition of the society and may also be considered a possible vehicle for delivery of probiotics to these communities and a source of genetically diverse strains with probiotic properties in general. *Kimere* is consumed without heat treatment after fermentation and hence contains 'live' bacteria. We have shown 10^8 colony forming units (CFU) of lactobacilli to be present per gram of *Kimere* (Njeru et al., 2010), corresponding to a daily intake of approximately 5 x 10^{10} lactobacilli, assuming a daily intake of 500 g of gruel. Using molecular biology methods including PFGE we have isolated and characterized 48 *Lactobacillus* strains from *Ki mere.* Our previous *in vitro* study (Njeru et al., 2010) showed that some of the *L. fermentum* strains isolated were resistant to low pH and bile salts. Based on these results, in the first part of the present study we evaluated the immune-modulating effects of these strains on the expression levels of the Th1 and Th2 cytokines IFN-! and IL-4, respectively, in human PBMCs in response to SEA superantigen. Although previous *in vitro* and *in vivo* studies have shown that modulation of production of cytokines by probiotics in human and animal models is dose, time, and particularly strain dependent (FAO/WHO, 2002; Fujiwara et al., 2004; Ghadimi et al., 2008; Kekkonen et al., 2008; Kopp et al., 2008; Luyer et al., 2005; Maassen et al., 2008; Pochard et al., 2002; Ryan et al., 2008), there seems to be no study so far that has compared *L. fermentum* strains. In the second part of the study the molecular basis for induction of the Th1-response (IFN-!) was investigated by Suppression Subtractive Hybridisation (SSH) (Annunziato et al., 2007; Ghadimi et al., 2011). We constructed subtracted cDNA libraries for *L. fermentum* strains K1-Lb1 (Th1-stimulating strain) and K8-Lb1 (Th1-nonstimulating strain) in order to identify genes potentially involved in modulation of the Th1 response in PBMCs. Finally, we assessed the hydrophobicities of the cell surfaces of strains K1-Lb1 and K8-Lb1, respectively, in order to verify whether genetic differences of these strains

resulted in alterations of their surface structures. In this context, adhesion of both strains to intestinal HT-29 cells was assessed, too.

2 Material and Methods

2.1 Bacterial strains

47 strains of *L. fermentum* and one *Lactobacillus plantarum* strain were isolated from various *Kimere* samples, collected from 11 different homesteads in the Mbeere community of Kenya and characterized using classical microbiological and molecular biology methods (Njeru et al., 2010et al). Briefly; *Lactobacillus* isolates were characterized and identified using biochemical methods like carbohydrate fermentation patterns, API 50 CHL, growth temperatures, and Gram and catalase reaction, as well as molecular methods like species-specific polymerase chain reaction (PCR), amplified rDNA restriction analysis (ARDRA) and partial sequencing of 16S rDNA. To study strain diversity, 46 *L. fermentum* isolates were subjected to pulsed-field gel electrophoresis (PFGE) analysis, using AscI as restriction enzyme. Analysis of *L. fermentum* strains with PFGE indicated different profiles and relatively large biodiversity within that species for 38 strains. Eight strains were excluded from further evaluation due to unsatisfactory PFGE profiles. Remaining bacterial strains were maintained at -80°C in MAST Cryobank™ (Mast Diagnostic-Reinfeld Germany) and among them, based on bile salt tolerance, the following strains were selected and used in this study: *L. fermentum K1-Lb1, L. fermentum K1-Lb6, L. fermentum K2-Lb4, L. fermentum K6-Lb2, L. fermentum K6-Lb4, L. fermentum K7-Lb1, L. fermentum K8-Lb1, L. fermentum K8-Lb3, L. fermentum K9-Lb3,* and *Lactobacillus plantarum K4-Lb6.*

2.2 Propagation of bacteria

Bacterial strains were propagated according to previously published procedures (Ghadimi et al., 2008). Briefly, using a 0.02 % inoculum from bacterial stocks stored at –80 °C in 30 % glycerol, lactobacilli were grown in MRS broth medium (according to de Man, Rogosa, Sharpe; Merck,

Darmstadt, Germany) anaerobically (The Modular Atmosphere Controlled System, MACS-VA500 workstation with airlock, Don Whitley Scientific Limited UK) at 37 °C overnight. Bacterial cultures were centrifuged at 14,000 x g (approximately 14,500 rpm in Eppendorf Minispin-plus centrifuge) for 2 min. Bacterial pellets were washed two times with phosphate-buffered saline (PBS), suspended in 1ml PBS containing 20 % glycerol, counted in a counting chamber (Neubauer improved), adjusted at concentrations of 10^8 cells/ml in PBS solution containing 20 % glycerol and stored at -80 °C until use. *Lactobacillus rhamnosus* GG (ATCC 53103) a strain known to stimulate IFN-γ and *Escherichia coli* TG1 (BU-00035) a strain known to stimulate IL-4 (Ghadimi et al., 2008; Pochard et al., 2002) were used as positive controls for induction of expression of Th1 and Th2 cytokines, respectively. *E. coli* TG1 was grown aerobically overnight at 37 °C in Luria–Bertani (LB) broth.

2.3 Assessing immunomodulatory effects on the protein expression levels of *Th1*-(IFN-γ) and Th2-(IL-4) cytokines

2.3.1 PBMCs preparation

Healthy donors

Blood (100ml) was collected from healthy donors, aged between 21-52 years. The selection criterion was based on exclusion of subjects who reported to have history of allergy, recent upper respiratory infection, flu or to be in medication. Recruitment and blood sampling followed strict ethical considerations laid down by the Ethics Committee of the University of Kiel on the Use of Human Subjects in Research. Informed written consent was obtained from all subjects prior to their enrollment in this study. The tube contents were inverted (8 – 10 times) to ensure that the whole blood was mixed thoroughly with the anti-coagulant (EDTA). Samples were stored at room temperature (18-25^{o}C) before PBMCs isolation and PBMCs were prepared according to previously published procedures (Ghadimi et al., 2008). Briefly, venous whole blood from healthy subjects was drawn into heparinized vacutainers and diluted with the same volume of pyrogen-free 0.9 % NaCl. The PBMCs were then isolated by density gradient

centrifugation (1.077 g/ml) (Lymphoprep, AXIS–SHIELD PoC AS, Oslo, Norway) and washed twice in endotoxin-free DPBS without Ca^{2+} and Mg^{2+}, containing 5 % foetal bovine serum (FBS; GIBCO). All PBMCs were >95% viable immediately after purification as assessed by microscopic examination of trypan blue exclusion.

2.3.2 Co-incubation of PBMCs and bacteria

PBMCs (2×10^6 cells/well/ml) were seeded in duplicate into 24-well tissue culture plates (Corning, Sigma) and co-incubated with bacteria (2×10^7 CFU/well/ml) at 37 °C in a humidified atmosphere with 5% CO_2 for 48 hours. The bacteria-to-cell ratio of 10:1 was chosen based on our own and other previous studies (Ghadimi et al., 2008; Ghadimi et al., 2010; Ghadimi et al., 2011; Ghadimi et al., 2012; Kekkonen et al., 2008; Pochard et al., 2002).

Besides basal state (medium), a stimulatory state was assessed using *Staphylococcus* enterotoxin A (SEA) at a concentration of 1 µg/ml. Treatments were: PBMCs + medium (control), PBMCs + SEA + medium, PBMCs + bacteria + medium, and PBMCs + SEA + bacteria + medium. Treatments were done in duplicates in 1ml volume in a 24-well plate. After incubation at 37 °C in humidified atmosphere with 5% CO_2 for 48 hours, supernatants were centrifuged at 179.5 x g and 4°C for 10 min, filtered through sterile micro filters (0.2 µm) into Eppendorf tubes (1.5 ml) in volumes of 250 µl and stored at -20 °C before subjected to ELISA tests.

2.3.3 Determination of cytokine protein levels

Levels of the IFN-! and IL-4 proteins in cell-free supernatants were quantified using specific human IFN-! and IL-4 ELISA Development Kits (Mabtech AB, Hamburg, Germany). Detection limits of the assays were 2 pg/ml for IFN-γ and 1 pg/ml for IL-4. Optical density values of the samples were read at 450 and 570 nm on an ELISA plate reader (Molecular Devices, Munich,

Germany). Each ELISA was performed in triplicate with cell-fee supernatants from 13 healthy subjects, and each result was expressed as the mean value of 13 subjects ! SEM.

2.4 Constructing subtracted cDNA libraries using SSH

Subtracted DNA libraries for *L. fermentum* strains K1-Lb1 and K8-Lb1 were generated using the PCR-Select™ Bacterial Genome Subtraction Kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France), as described previously (Ghadimi et al., 2011). However, HaeIII- instead of RsaI-digested genomic DNA was used as "tester" and ''driver'' (Diatchenko et al., 1996), respectively. Differential cDNA fragments were directly cloned into pSTBlue-1 cloning vector and subjected to blue/white screening. Dot blot analysis was applied for recombinant plasmids to identify strain-specific cloned fragments. 20 K1-Lb1- and 10 K8-Lb1-specific clones were randomly selected and sequenced. To determine putative functions of potentially expressed proteins, DNA sequences obtained were analyzed as described previously (Altschul et al., 1990; Ghadimi et al., 2011), using the ''BLASTN'' and ''BLASTX'' algorithms of the National Centre for Biotechnology Information (NCBI) (http://blast.ncib.nlm.nih.gov/Blast). Similarities between sequences of different clones were analyzed by ClustalW at http://align.genome.jp/.

2.5 Surface hydrophobicities of *L. fermentum* strains

The test for bacterial adhesion to hydrocarbons (Doyle and Rosenberg, 1995) was adopted. 10 ml of cultures of *L. fermentum* strains grown in MRS-medium overnight were sedimented by centrifugation at 9,500 x g and washed twice with Ringer solution before re-suspended in Ringer solution. Optical density was measured at 580nm (OD1). Each suspension (1.5 ml) was mixed with an equal volume of n-hexadecan and vortexed for 2 min. After allowing phase separation for 30 min at room temperature, 1 ml of the lower aequeous phase was withdrawn and OD_{580nm} was measured (OD2). Relative hydrophobicity was calculated as follows: % hydrophobicity = [(OD1 – OD2) / OD1] x 100. Measurements were done in triplicate.

2.6 Intestinal epithelial cell culture conditions

HT-29 human colon adenocarcinoma epithelial cells (DSMZ, ACC 299) were grown in antibiotic-free Dulbecco's modified Eagle's essential medium supplemented with 10 % foetal calf serum (Invitrogen, Eggenstein, Germany) at 37°C in 10% CO_2 atmosphere.

2.7 Adhesion of *L. fermentum* strains to human HT-29 colonic epithelial cell line

4 x 10^5 HT-29 cells per well were placed into 24-well tissue culture plates and incubated for 4-5 days until a monolayer had formed. Incubation was done in Dulbecco's modified eagle medium supplemented with 10 % foetal calf serum. Cultures of lactobacilli were grown in MRS medium overnight, centrifuged, washed with PBS and taken up in PBS. Determination of CFU was done by plating serial dilutions onto MRS agar plates. After adding 1 ml of bacterial suspension (1 x 10^8 CFU/ml) to each of the wells containing HT-29 monolayers, the plates were centrifuged at 2,000 x g for 2 min. After incubation at 37°C for 1 h, the supernatants were withdrawn and discarded. The monolayers were washed two times, followed by lysis of the cells with Triton X-100 (0.05%). CFU of lactobacilli were determined on MRS agar plates. Relative adhesion was calculated by dividing the CFU obtained after lysing of the monolayers by the CFU added to the monolayers and multiplying with 100. Adhesion experiments were always carried out in triplicate.

2.8 Statistical analysis

Statistical analyses were performed using STATGRAPHICS Plus statistical software—Version 4.1. Data were subjected to analysis of variance (ANOVA). Because of a non-normal distribution of most of the data the nonparametric Mann–Whitney *U*-test was used to compare cytokine data. This test allowed comparing data from cultures in the absence of a bacterial strain with cultures in the presence of the different strains. In case of the cytokines data, all experimental data were expressed as the standard error of the mean (mean ! SEM) and were considered statistically significant when $p < 0.05$. In the case of surface hydrophobicities and

adhesion of *L. fermentum* strains K1-Lb1 and K8-Lb1 to HT-29 cells, all experiments were carried out in triplicate and data are expressed as mean ! SEM.

3. Results

3.1 Cytokine production patterns

As shown in **Figures 1A**, **B**, and **C,** strain-specific effects on cytokine secretion by PBMCs was observed among nine *L. fermentum* strains and one *L. plantarum*. Two strains, K1-Lb1 and K4-Lb6, significantly induced basal IFN-! secretion. Four strains, K1-Lb6, K6-Lb2, K7-Lb1, and K8-Lb1 suppressed IFN-! secretion of SEA-stimulated PBMCs. All strains, except K1-Lb1, K2-Lb4, and K9-Lb3, significantly inhibited SEA-stimulated IL-4 secretion. Overall, these data show that, in both basal and SEA-stimulated PBMCs, some *Kimere* strains (K1-Lb1, K2-Lb4, K4-Lb6, and K9-Lb3) shifted the immune system (IFN-!:IL-4 ratio) towards a Th1 response when compared with control cell cultures (**Figure 1C**). As expected, the *E. coli* TG1 strain, applied as negative control, had no effect on secretion levels of either basal or SEA-stimulated INF-!. Although this strain had no significant effect on the secretion level of basal IL-4, it significantly induced IL-4 production in SEA-stimulated PBMCs. The probiotic *L. rhamnosus* GG (LGG), applied as positive control, significantly induced IFN-! secretion in both unstimulated and SEA-stimulated PBMCs. As expected, this strain significantly inhibited IL-4 secretion from SEA-stimulated PBMCs. The basal and SEA stimulated Th1/Th2 ratios significantly differed between K1-Lb1 and K8-Lb1 ($p < 0.05$). Therefore, these two strains were selected for identifying candidate genes mediating immunomodulation.

3.2 Suppression subtractive hybridization (SSH) using K1-Lb1 and K8-Lb1 genomic DNAs

Success of subtraction of genomic DNAs of *L. fermentum* strains K1-Lb1 (exhibiting a Th1/Th2 shift towards Th1 in both untreated and SEA-treated PBMCs) and K8-Lb1 (exhibiting

suppression of Th1 and Th2 and no shift in Th1/Th2 ratio) became obvious by the presence of distinct bands among the background of non-subtracted PCR products (**Figure 2**). After purifying the subtracted libraries, cloning into pSTBlue-1 vector and screening for white colonies, 110 individual clones were randomly selected from reciprocal subtractions. Digestion of clones with EcoRI confirmed the presence of inserts with sizes ranging from about 150 bp to more than 1000 bp (data not shown). By dot blot analysis, strain specificity was confirmed for the inserts of many of the clones (**Figure 3**), from which 20 specific for K1-Lb1 and 10 specific for K8-Lb1 were randomly selected and sequenced.

When screening the sequences for presence of HaeIII-sites (the enzyme used for restriction of the genomic DNAs) in order to identify the cloned PCR products, we found that several of the recombinant plasmids appeared to contain more than one PCR-product. In BLASTX analyses all 20 clones specific for K1-Lb1 and all 10 clones specific for K8-Lb1 contained DNA inserts showing variable homologies to known proteins or genes in the databases (**Table 2**). Only DNA sequences, which were detected more than once among the 20 or 10 clones, respectively, were considered significantly different from the respective driver DNA. Most DNA inserts showed high homologies to *L. fermentum* sequences.

Using K1-Lb1 DNA as tester DNA, a DNA fragment with up to 95 % homology to a putative glycosyltransferase of *L. fermentum* 14391 was found 5 times among the twenty clones sequenced. In all of these clones, the other part of the sequence coded for a putative polysaccharide pyruvyl transferase. Homology was not very high (<48%), but the E-value indicated significance. In three further clones, highest homology to the same putative polysaccharide pyruvyl transferase was found, indicating that another part of the same gene had been cloned. It is interesting to note that part of a gene, coding for a transposase, was located on some of the cloned insert. Two cloned inserts showed sequence homologies to a protein (NorM) potentially involved in multi-drug export, and two further clones showed sequence homology to a family S9 peptidase gene. Finally, two and three clones revealed the presence of part of a transposase gene. Only four of the twenty DNA inserts had been picked

up once (i.e. showing no DNA homology to any of the other cloned inserts), demonstrating the efficiency of the SSH method.

Using K8-Lb1 DNA as tester DNA, only two pairs of clones, 02, 09 and 07, 10, revealed identical DNA sequences. They showed homologies to an integrase gene, indicating chromosome alterations possibly originating from insertion of a prophage, and to hypothetical proteins. All other inserts shown in **Table 2** were picked up only once. In contrast to K1-Lb1, no DNA sequences encoding potential surface associated functions were found in K8-Lb1.

3.3 Surface properties and adhesion to HT-29 cells

Based on the SSH results, K1-Lb1 and K8-Lb1 appeared to differ in enzymes involved in polysaccharide synthesis. This apparently affected their surface properties (**Figure 4).** Both strains differed considerably in surface hydrophobicity with K1-Lb1 exhibiting very low (ca. 5%) and K8-Lb1 very high (ca. 80%) hydrophobicity. However, both strains showed similar adhesion to HT29 cells at a level of ca. 10%.

4. Discussion

As described in a former publication (Njeru et al., 2010), 10 lactobacilli strains isolated from *Kimere* (nine *L. fermentum* strains and one *L. plantarum*) were examined for their tolerance to bile salts and survival under low pH. All strains tested were tolerant to 0.3% ox gall, with *L. plantarum* strain K4-Lb6 showing tolerance up to 3% ox gall. Strains K7-Lb1 and K8-Lb1 showed survival rates at pH 2.0 of 59% and 22%, respectively, while most other strains were able to survive pH 3.0. Tolerance to pH 3.0 appears to be sufficient for strains to be applied as probiotics, since the most common application of probiotics is in dairy products, which are known for their considerable buffering capacities. Tolerance to bile salts is another important criterion for coping with the adverse gastrointestinal conditions.

Evaluation of the immunomodulating properties of the *Kimere* strains resistant to bile salts and low pH showed different outcomes. Several strains shifted PBMCs towards Th1 response, when compared with control cell cultures with and/or without addition of SEA superantigen. These data illustrate the potential of *L. fermentum* strain K1-Lb1 and *L. plantarum* strain K4-Lb6 to influence, in a strain-specific manner, the Th1/Th2 balance by shifting it to a Th1 direction and to modulate aberrant immune response(s) to SEA superantigen. In comparison to LGG, this shift, however, was less pronounced. Some other *L. fermentum* strains (K1-Lb6, K6-Lb2, K7-Lb1, and K8-Lb1) reduced both the Th1- and Th2-response to SEA, which may imply anti-inflammatory potential. In principal, final conclusions on health effects in humans cannot be drawn from *in vitro* data. *In vitro* testing PBMC cytokine response, however, has been shown to enable predictions of *in vivo* inflammatory outcomes (Foligne et al., 2007). Thus, our results may indicate probiotic potential for the new *L. fermentum* strains isolated from the African fermented food *Kimere*.

There is growing evidence from *in vitro* and *in vivo* studies, with animal models and humans, that probiotic bacteria may reduce Th1 or Th2 skewed disorders, like inflammatory bowel disease and allergic diseases and that their effects on Th1/Th2 cell modulation are strain-specific (Ghadimi et al., 2008; Gillingham and Lescheid, 2009). Certain probiotic strains can influence secretion of cytokines to help naïve *T*-helper cells to develop toward either a Th1-dominant, cell-mediated immune response or toward a Th2-dominant, humoral immune response; several probiotics were reported to help balance these immune responses (Shandilya et al., 2011; Winkler et al., 2007). The molecular basis of such strain-specific effects of probiotic bacteria, however, is still not well understood. Therefore, using SSH technique we constructed subtracted cDNA libraries for *L. fermentum* strains K1-Lb1 (IFN-! stimulating, Th1/Th2 shifting towards Th1 strain) and K8-Lb1 (IFN-!-suppressing strain) to find out which differences in their bacterial genomes may be responsible for the different effects of these strains. The rationale for genetic comparison of these strains was that although K1-Lb1, K2-Lb4, K4-Lb6 and K9-Lb3 enhanced both basal and SEA-stimulated IFN-!:IL-4 ratio, K1-Lb1 was the better inducer of

basal IFN-!:IL-4 ratio and the difference between K1-Lb1 (as inducer) and K8-Lb1 (as inhibitor) was significant. In contrast, the difference between e.g. K9-Lb3 and K8-Lb1 was not significant. As indicated by dot-blot hybridization, subtraction of K1-Lb1 DNA with K8-Lb1 DNA yielded more K1-Lb1-specific clones than subtraction of K8-Lb1 DNA with K1-Lb1 DNA. Apparently, K1-Lb1 seems to harbour more genes not present in K8-Lb1 than vice versa. This was confirmed by our sequencing results. For K8-Lb1 only two specific genes were found, a gene encoding a phage integrase which was picked up twice, and two genes encoding hypothetical proteins also picked up twice. The presence of an integrase gene may indicate presence of a prophage in K8-Lb1 not found in K1-Lb1. This is not surprising, since phages are known to have pronounced host-specificities (Brüssow et al., 1998) and are also known to be involved in horizontal gene transfer playing a role in strain adaptability and evolution (Canchaya et al.; 2003, Mahillon and Chandler, 1998 ; Zago et al., 2007). Several clones contained DNA encoding different transposases, however, each DNA encoding a specific transpose was only picked up once. Although this does not allow for categorizing these clones as K1-Lb1-specific, the occurrence of transposase genes can be expected, since transposases are involved in genome arrangements and strain evolution (Mahillon and Chandler, 1998 and Mahillon et al., 1999).Thus, it is foreseeable that different strains of a species differ in copy numbers, locations and types of transposases.

Among the 20 cloned K1-Lb1 DNA inserts sequenced, 16 were found to be K1-Lb1-specific according to the criterion that sequences have to be picked up more than once to be named strain-specific. Seven clones contained DNA encoding a hypothetical polysaccharide pyruvyl transferase (PPT) involved in exopolysaccharide (EPS) synthesis (Peant et al., 2005). Inserts in clones K1-C03, K1-C04, K1-C08, K1-C11 and K1-C12 were found to code for a protein with highest homology to a putative glucosyltransferase (GTF) of *L. fermentum* ATCC 14931. GTFs catalyze the synthesis of simple and complex carbohydrates by sequentially adding UDP-glucose from activated donors to target acceptors by forming glycosidic bonds (Schuman et al., 2007). Acceptor molecules can be lipids, proteins, heterocyclic compounds, or other

carbohydrate residues. Hence, they can be termed as being involved in the biosynthesis of polysaccharides, glycoproteins and glycolipids.

All five inserts harboring part of a GTF gene also harbored part of a gene potentially encoding a PPT. Three clones (C03, C11, C13) and two clones (C04 and C08), respectively, carried identical DNA inserts. The parts of the sequences encoding PPT differed between the two groups and were not overlapping. Since the deduced amino acid sequence homologies of the putative PPTs were rather low at around 40%, it is not clear, whether all five clones code for the same protein with similarity to PPT. Also, due to the low similarity, a corresponding gene has not yet been identified in lactobacilli. Together with the fact that clones C13 and C16 also carry part of the same transposase gene, this may argue in favour of *L. fermentum* K1-Lb1 having received the PPT gene by horizontal gene transfer.

K1-Lb1-specific clones C05 and C10 both carried the same DNA fragment encoding a protein with highest similarity to an *Enterococcus faecium* protein exhibiting the conserved domain NorM (Brown et al., 1999). The latter is described as potentially acting as Na^+-driven multidrug efflux pump. However, BLAST analysis revealed a conserved domain RfbX, indicating involvement in export of O-antigen and lipoteichoic acid (Morita et al., 2009). All these data on K1-Lb-1-specific genes suggest that strain K1-Lb1 synthesizes specific polysaccharides, which are not synthesized by strain K8-Lb1. In principle ,oligo- or polysaccharides may be part of inner structures, of the cell membrane or of the cell wall, or they may be released as exopolysaccharides (EPS). The pronounced difference in hydrophobicity of the cell surfaces of both strains, however, suggests that they differ in surface structures.

Presence of unique polysaccharide structures on the surface of strain K1-Lb1 is supported by the cloning - although picked up only once - of two DNA fragments (C02, C19) potentially coding for an acyltransferase and a polysaccharide biosynthesis protein and for a sugar transferase and a UDP-glucose-4-epimerase, respectively. The latter enzymes are involved in biosynthesis of peptidoglycan (PGN), whereas acyltransferases utilize fatty acid chains to form

in a first step membrane phospholipids. Thus, play a critical role in the regulation of membrane biogenesis through acyl-CoA or acyl-acyl carrier protein (Acyl-ACP) pathways (Hutchings et al., 2009; Zhang and Rock, 2008).

During biosynthesis of wall teichoic acid (WTA) and lipoteichoic acid (LTA), GTFs catalyze the transfer of UDP-Glc molecules into sugar acceptors forming glycosidic bonds (Neuhaus and Baddiley, 2003) This process is essential in the binding of glycosyl residues to the repeating glycerophosphate units of LTA chains. This implies that GTFs and acyltransferase are also essential in biosynthesis of a glycolipid that anchors LTA in cell walls of Gram-positive bacteria (Kiriukhin et al., 2001).

During PGN biosynthesis, GTFs also catalyze the transfer of N-acetylglucosamine (NAG) from UDP to the C4 hydroxyl group of a lipid-linked N-acetylmuramic acid (NAM) by forming a β-linked NAG-NAM disaccharide. The latter is transported across the membrane where it is polymerized and cross-linked with the help of other enzymes (Ha et al., 2000). PGNs are cell-wall polymers outside the plasma membrane consisting of sugars and amino acids. They form an important part of the cell-wall, giving structural shape and protection from changing environmental osmotic pressures. Being the outermost layer, they interact with the host immune competent receptors, e.g. TLR2 and peptidoglycan recognition proteins and hence play a major role in immune response. Cell surface structures such as PGNs, LTAs, WTA and other polysaccharides together with enzymes encoding for their biosynthesis play a major role in host-microbe interaction through microbe-associated molecular patterns (MAMPs) such as TLRs and NOD (Corthesy et al., 2007; Takeda and Akira, 2004; Winkler et al., 2007). Failure to encode the enzymes would most likely result in synthesis of modified PGNs, which may change their MAMP pattern.

Peptidoglycans and LTAs mediate increase of IFN-! and activity of NK cells (Makino et al., 2006; Vinderola et al., 2006). At gene level, Grangette et al. (2005) demonstrated that suppression of the *dlt* operon (*dlt*$^{-}$ mutant) affected the type of LTA produced and also changed

the immunomodulatory properties of *L. plantarum*. Their study showed that a defect in D-Alanine substitution of LTA changed the immune response from inflammatory to anti-inflammatory using human PBMCs. Hence disruption of the structure or lack of enzymes that catalyze its formation, would affect the immunological properties of the host.

Purified GTFs enzymes by themselves have been shown to be immunologically active, whereby they stimulate T-cell proliferation to produce pro-inflammatory responses (Chia et al., 2001). Studies involving GTFs from Gram-positive *Streptococcus mutans*, have demonstrated that GTFs act through activation of NF-κB resulting in IL-6, IL-8 and TNF-α stimulation (Chia et al., 2002; Shun et al., 2005; Yeh et al., 2006). Whether the response to commensal bacterial GTFs would be different from *S. mutans* GTFs still remains speculation. Anyway, their differences could imply strain specific immune responses.

Altered synthesis of polysaccharides may affect structure or quantity of EPS. EPS protects bacteria from environmental stresses and infection by bacteriophages and it interacts with host immune receptors by triggering various immune responses (Boels et al., 2001; Groot and Kleerebezem, 2007; Jolly et al., 2002). EPS has been shown to be structurally diverse and differ from one strain to another (Mahillon et al., 1999). Purified EPS of *L. delbrueckii* ssp *bulgaricus* strain OLL1073R-1 increased IFN-! production in mouse splenocytes and augmented NK-cells activities both *in vitro* and *in vivo*. Feeding mice with yoghurt fermented by this strain resulted in expression of similar cytokines as if fed with purified EPS (Makino et al., 2006). Hence, GTFs and cell surface structures could play a role in host-microbe interaction leading to the observed differences between the strains. Cell surface structures have been demonstrated to be involved in host-microbe interaction, adhesion and transient colonization in *L. johnsonii* (Granato et al., 1999) and also in *L. reuteri* (Roos and Jonsson, 2002). Furthermore, Yasuda et al. (2008) demonstrated by gene knock-out that cell wall components were involved in induction of cytokine production by *L. casei* strain Shirota, which was in agreement with earlier results by Grangette et al. (2005) using *L. plantarum* strains.

Thus, one is tempted to conclude that the observed differences in immunomodulatory properties between *L. fermentum* strains K1-Lb1 and K8-Lb1 could be due to the differences in one or more of the enzymes identified by SSH, since the expected catalytic products are part of cell surface structures known to play a major part in host-microbe interaction and in immunomodulation. Compared to K8-Lb1, K1-Lb1 may have altered composition or incorporated more cell-wall components, which may either better activate pattern recognition receptors or activate more receptors on PBMCs, which then contribute to production of both IFN-*!* and IL-4. In case of confirmation by gene expression studies, the molecular basis of bacterially mediated immune effects may be further elucidated and the identified gene(s) may be used for quality assurance of probiotic, immunomodulatory properties.

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Conflict of interest statement

K.J.H. and J.S. are inventors of a patent on some of the strains described in this article.

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Tables

Table 1: Oligonucleotides used in this study

Oligonucleotide	Sequence (5' . 3')	Description
Adaptor 1	5'- CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3' 3'-GGCCCGTCCA-5'	SSH adaptor
Adaptor 2R	5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3' 3'-GCCGGCTCCA-5'	SSH adaptor
Primer P1	5'-CTAATACGACTCACTATAGGGC-3'	Primary PCR
Nested Primer 1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'	Secondary PCR
Nested Primer 2	5'-AGCGTGGTCGCGGCCGAGGT-3'	Secondary PCR

Table 2: Blast analysis of strain-specific SSH clones

Clone(fragment)	length (bp)	Protein	Score (e-value[1]) %[2]	Identity /organism	Reference
K1-C13(b[3])	258	Hypothetical protein CLOSTMETH_01773	69.7 (1e-10) 40%	*Clostridium methylpentosum* DSM	ZP_03707031.1
K1-C16(b)	258	/ Polysaccharide pyruvyl transferase	69.7 (1e-10) 40%	5476	
K1-C20(b)	258	PS_Pyruv_trans (Pfam Acc.: PF04230)	69.7 (1e-10) 40%		
K1-C03(a)	426		130 (3e-28) 47%		
K1-C08(a)	426		130 (3e-28) 47%		
K1-C11(a)	426		130 (3e-28) 47%		
K1-C12(a)	426		130 (3e-28) 47%		
K1-C04(b)	318		61 (2e-08) 35%		
K1-C03(b)	471	Putative glycosyltransferase	301 (5e-87) 95%	*L. fermentum* ATCC 14931	ZP_03944259.1
K1-C11(b)	471		301 (5e-87) 95%		
K1-C12(b)	471		301 (5e-87) 95%		
K1-C04(a)	687		390 (3e-106) 84%		
K1-C08(b)	585		311 (1e-82) 82%		

K1-C05	957	Hypothetical protein; “NorM”	288 (4.8e-94) 48%	*Enterococcus faecium* E1636	ZP_06696133.1
K1-C10	951	(Na^+-driven multidrug efflux pump)	296 (4e-78) 48%		
K1-C01	63	Family S9 peptidase	57 (1e-04)100%	*L. fermentum* ATCC 14931	ZP_03945321.1
K1-C15	63		57 (1e-04)100%		
K1-C13(a)	123	Transposase	70.5 (7e-11) 86%	*L. fermentum* 28-3-CHN	ZP_05863524.1
K1-C16(a)	123		70.5 (7e-11) 86%		
K1-C20(a)	123		70.5 (7e-11) 86%		
K1-C06(a)	267	Transposase IS4 family protein	175 (3e-49) 99%	*L.* fermentum 28-3-CHN	ZP_05864732.1
K1-C07	267		172 (3e-49) 98%		
K1-C14	267		168 (3e-47) 97%		
K1-C09	831	Transposase	533 (0) 99%	*L. fermentum* ATCC 14931	YP_001843906
K1-C16(c)	141		58 (4e-08) 97%		

K1-C13(c)	141		58 (4e-08) 97%		
K1-C02(a)	429	Acyltransferase 3 (COG 3274: O-acyltrans-ferase)	269 (1e-85) 100%	*Lactobacillus fermentum* ATCC 14931	ZP_03943992.1
K1-C02(b)	282	Polysaccharide biosynthesis protein	183 (3e- 51) 98%	*L. fermentum* ATCC 14931	ZP_03943991.1
K1-C06(b)	96	Hypothetical protein	69 (5e-13) 97%	*Lactobacillus mali KCTC 3596*	ZP_09449485.1
K1-C17	327	Hypothetical protein	219 (1e-58) 99%	*L. fermentum* IFO 3956	YP_001843839
K1-C18	75	Possible transposase	57 (3e-07) 100%	*L. fermentum* ATCC 14931	ZP_03945788.1
K1-C19(a)	261	Sugar transferase	103 (5e-21) 68%	*Lactobacillus buchneri* NRRL B-30929	YP_004385937.1
K1-C19(b)	153	UDP-glucose-4-epimerase	77 (9e-15) 63%	*Lactobacillus buchneri* NRRL B-30929	YP_004385938.1

K8-C02	565	Integrase	380 (8e-130) 98%	*L. fermentum* 28-3-CHN	ZP_005864559.1
K8-C09	565				
K8-C07(b)	225	Predicted protein	150 (4e-42) 89%	*L. fermentum* 28-3-CHN	ZP_05864214.1
K8-C10(a)	225		150 (4e-42) 89%		
K8-C07(a)	402	Hypothetical protein	62 (1e-09) 30%	*Sporosarcina newyorkensis* 2681	ZP_08680453.1
K8-C10(b)	402		62 (1e-09) 30%		
K8-C01	357	Hypothetical protein	103 (8e-25) 54	Uncultured actinobacterium HF0500-35G12	ADI22944.1
K8-C03	905	Integrase	575 (0) 93%	*L. fermentum* ATCC 14931	ZP_03944306
K8-C04	681	Chromosome partitioning ATPase	511 (2e-179) 98%	*Lactobacillus hildgardii* ATCC 8290	ZP_03954197.1

K8-C05	6	YhgE protein	45 (9e-05) 100%	*L. fermentum* 28-3-CHN	ZP_5863261.1
K8-C06(a)	303	Transposase	193 (6e-73) 93%	*L. fermentum* 28-3-CHN	ZP_05864757
K8-C06(b)	153	Transposase	109 (9e-37) 97%	*L. fermentum* CECT 5716	YP_005848263
K8-C08(a)	336	RNA methyltransferase TrmH family	223 (8e-69) 99%	*L. fermentum* 28-3-CHN	YP_05864124.1
K8-C08(b)	168	RNA polymerase sigma factor	123 (2e-32)100%	*L. fermentum* 28-3-CHN	ZP_05864123.1

[1] The *e* value indicates the probability of the match. An e value < e^{-5} of an alignment means that it is highly significant and not due to error or chance. The score is a measure of the similarity of the query to the sequences shown.

[2] % expresses identity/homologies to known proteins or genes in the databases.

[3] Letters a, b and c indicate different DNA restriction fragments cloned in one plasmid.

Figure legends

Figure 1: Effect of *L. fermentum* strains isolated from *Kimere* on cytokine protein expression levels of Th1-IFN-! (**A**), Th2-IL-4 (**B**), and Th1-IFN-! to Th2-IL-4 ratio(**C**) in response to *Staphylococcus enterotoxin A* (SEA) superantigen by human PBMCs. Human PBMCs (1 x 10^6 cells/ml) were co-incubated with bacterial strains (2 x 10^7 cells/ml) with and without stimulation with SEA (1µg/ml) at 37 °C for 48 hours. Supernatants were analyzed for cytokines by ELISA. Data are expressed as mean ± SEM (n = 13) of cytokines concentrations. Asterisk (*) means significantly different when compared with control cell cultures with addition of SEA, $p<0.05$, Mann-Whitney *U*-test. Paragraph (§) means significantly different when compared with control cell cultures without addition of SEA, $p<0.05$, Mann-Whitney *U*-test. Pi symbol (!) means significantly different when compared with K1-Lb1, $p<0.05$, Mann-Whitney *U*-test.

Figure 2: Subtracted secondary PCR products. PCR products were resolved by electrophoresis on a 1% agarose gel, stained with ethidium bromide and observed under UV light. Lanes: M, λ-DNA marker; lane 1, subtracted K1-Lb1; lane 2, subtracted K8-Lb1; lane 3, unsubtracted K1-Lb1; lane 4, unsubtracted K8-Lb1.

Figure 3: Dot blotting for screening tester specific inserts using HaeIII-digested tester and driver DNAs in reciprocal subtractions. Panel **a)** K1-Lb1 specific clones with K1-Lb1 as the probe; panel **b)** K1-Lb1 specific inserts with K8-Lb1 as the probe; panel **c)** K8-Lb1 specific[0] clones with K8-Lb1 as the probe; panel **d)** K8-Lb1 specific[0] clones with K1-Lb1 as the probe. Blots enclosed by a circle are those which were randomly selected and sequenced.

Figure 4: Surface hydrophobicities (grey bars) and adhesion to HT-29 cells (black bars) of *L. fermentum* strains K1-Lb1 and K8-Lb1, respectively. All experiments were carried out in triplicate. Data are expressed as mean ± SEM. Number sign (#) means significantly different when compared with *L.fermentum* K1-Lb1, $p<0.05$, Mann-Whitney *U*-test.

Figures

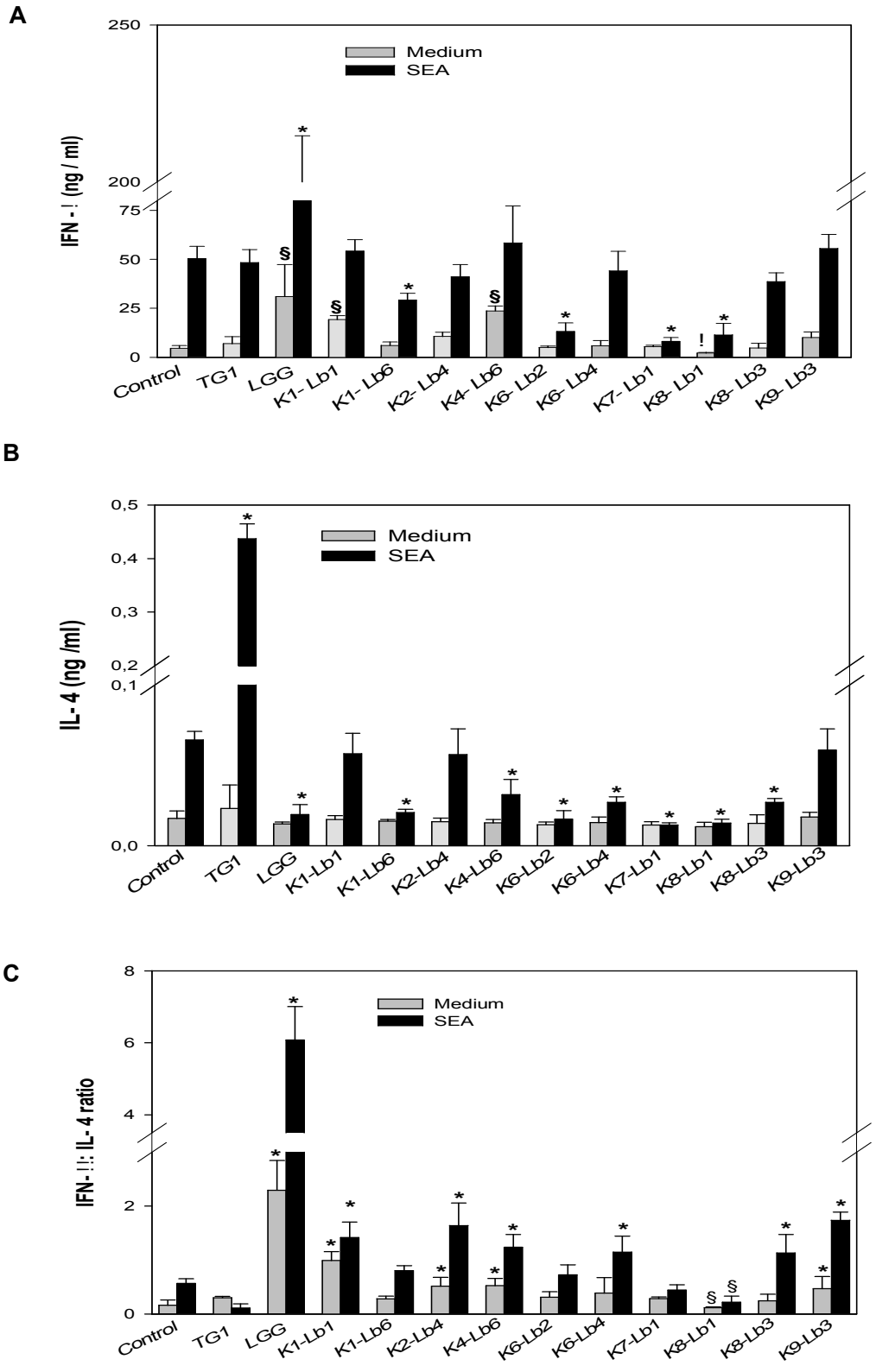

Figure 1 : Ghadimi et al.

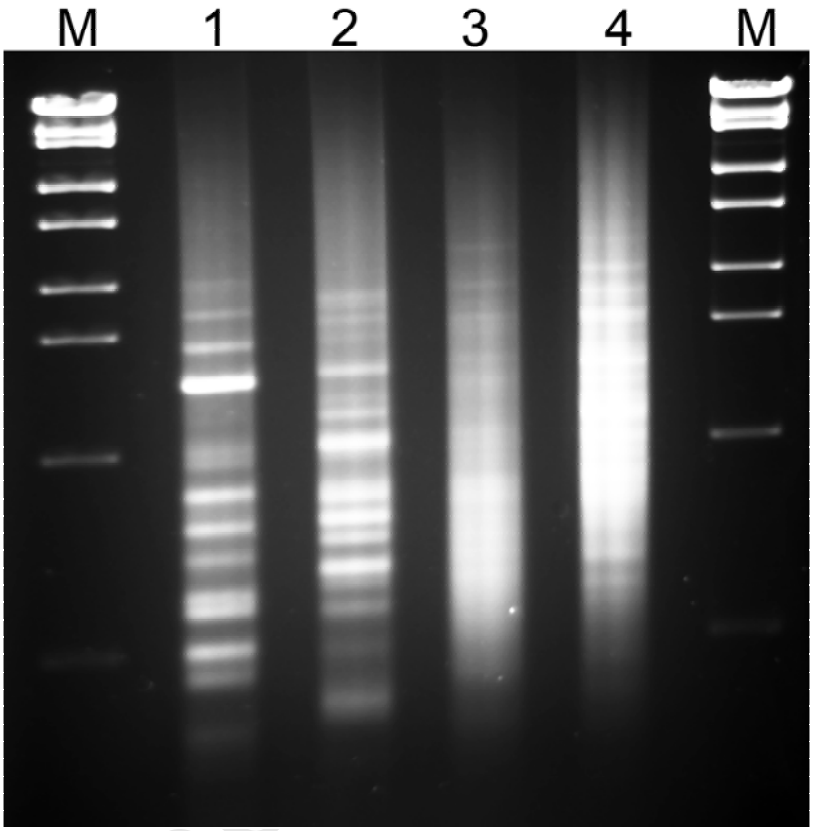

Figure 2: Ghadimi et al.

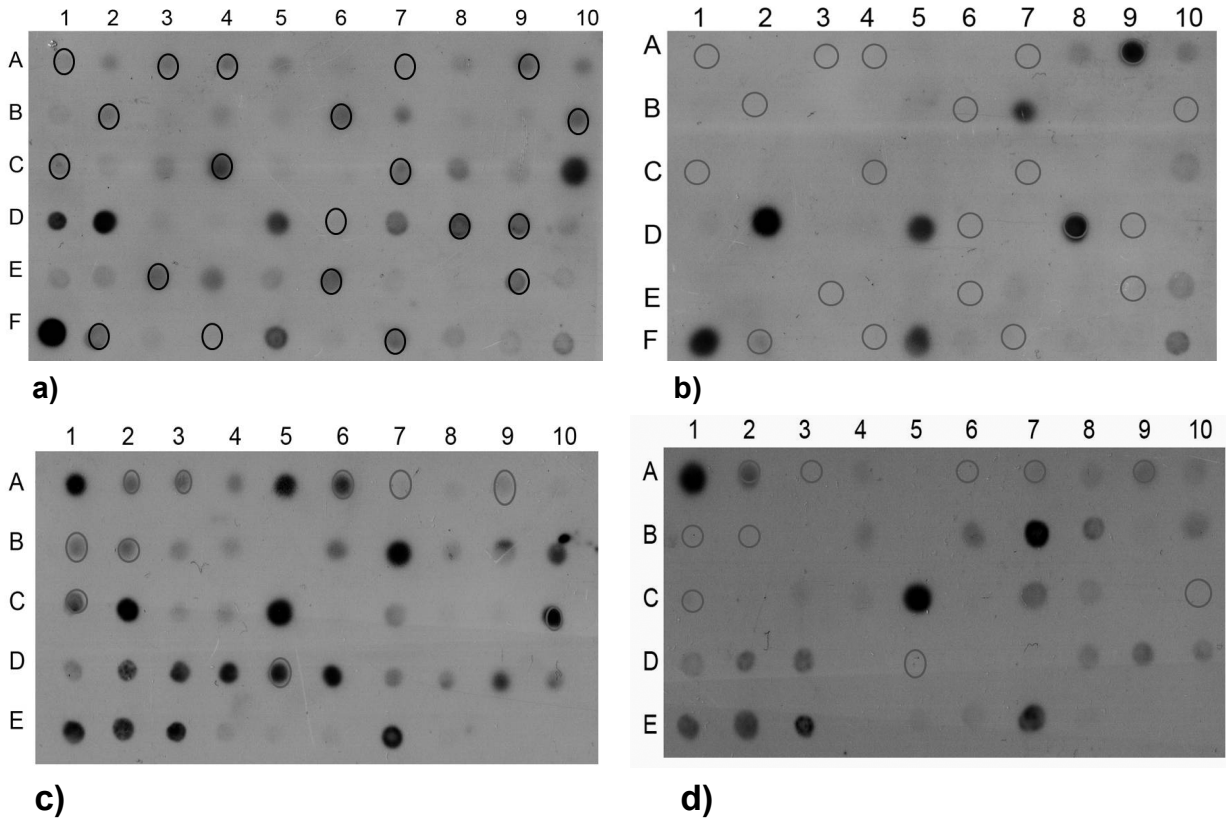

Figure 3: Ghadimi et al.

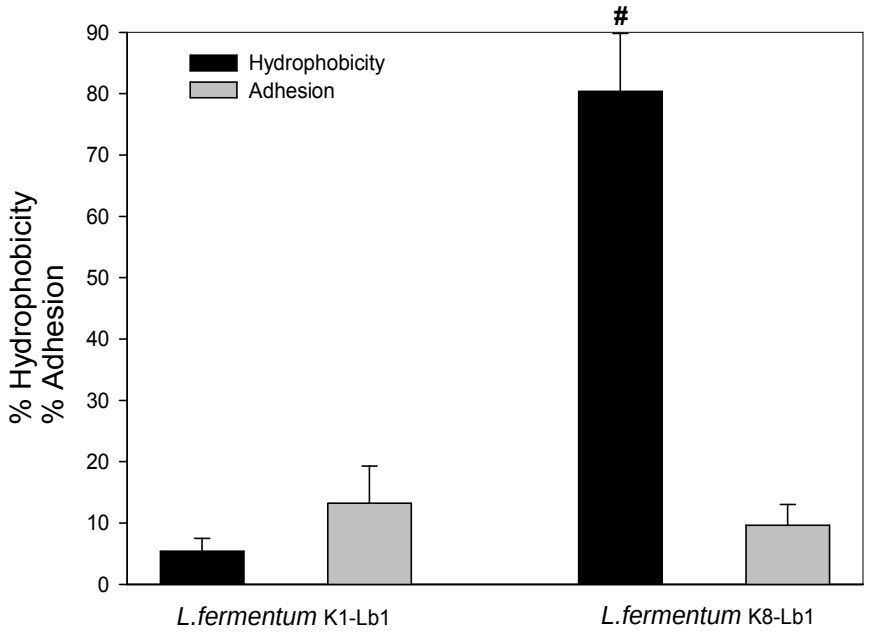

Figure 4: Ghadimi et al*.*