



## Autoinducer-2 associated inhibition by *Lactobacillus sakei* NR28 reduces virulence of enterohaemorrhagic *Escherichia coli* O157:H7



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### ABSTRACT

Bacteria use quorum sensing (QS) to regulate the expression of certain target genes for social behaviour. A LuxS/AI-2 signalling system serves to control the virulence of some pathogenic bacteria by mechanisms such as motility, biofilm formation and attachment, and is typical of the enterohaemorrhagic *Escherichia coli* O157:H7 (EHEC) associated with infections of the human intestine. The LuxS/AI-2 signalling system presents an interesting potential as antimicrobial target for appropriate AI-2 inhibitors, and thus widens the scope for treatment or prevention of infections by pathogens such as EHEC. Probiotic lactic acid bacteria (LAB) are primary candidates for this approach because of their general acceptability, safety and adaptation to the intestinal and/or food ecosystem. In this paper, we report on *Lactobacillus sakei* NR28 as a new candidate strain for AI-2 related quorum quenching. It is considered to be a putative probiotic strain and was originally isolated from kimchi, a traditional Korean fermented food known for its special health features. This study has shown that AI-2 activity and the associated virulence factors of the EHEC 'wild-type' strain *E. coli* ATCC 43894, were significantly reduced by *L. sakei* NR28, while, at the same time, the cell viability of the EHEC strain was not affected. In addition, the purified AI-2 molecule, a *luxS*-deficient mutant of EHEC strain ATCC 43894, and an AI-2 independent EHEC mimicking strain of *Citrobacter rodentium* were used to determine the relationship between the virulence reducing effect of *L. sakei* NR28 and its AI-2 inhibiting ability. Our results showed that *L. sakei* NR28 has a reducing effect on the pathogenicity of the 'wild-type' EHEC strain ATCC 43894 by AI-2 signalling inhibition.

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### 1. Introduction

Quorum sensing is a cell density dependent bacterial communication system (Xavier & Bassler, 2005a). The role of this signalling system is to regulate social behaviour of bacteria by intra/interspecies and inter-kingdom interaction such as biofilm formation, specific metabolite production, endospore-formation, cooperation and competition (Miller & Basler, 2001). Therefore, understanding bacterial quorum sensing may serve to support industrial applications of this principle for food preservation and the development of new antibiotics (Chen et al., 2011).

Under "natural" conditions, single strain bacterial association with a niche or ecosystem will practically never be encountered.

Therefore, typical of mixed culture conditions, bacteria have species/strain-specific quorum sensing signal systems for communication. However, it has been shown that bacteria possess not only an intra-species but also an inter-species signalling system (Bassler & Losick, 2006; Hughes & Sperandio, 2008). One of the primary bacterial inter-species communication mechanisms is by the *luxS*-mediated universal signalling system using autoinducer-2 (AI-2) as signalling molecule (Xavier & Bassler, 2005b), with a *luxS* homologue typical of multiple bacterial species (Pereira, Thompson, & Xavier, 2012). For example, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* use the LuxS/AI-2 signal for regulation of biofilm formation (Miller & Basler, 2001; Pereira et al., 2012; Xavier & Bassler, 2005a). In *Campylobacter jejuni*, the AI-2 signal is used to control motility (Elvers & Park, 2002), whereas *Streptococcus mutans* regulates stress tolerance related gene expression by signalling (Wen & Burne, 2004). This mechanism has been found even in some *Lactobacillus* spp., and *Lactobacillus acidophilus* was reported to promote its intestinal adhesion ability and

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**Table 1**

Antimicrobial activity on LB agar plates of *Lactobacillus sakei* NR28 against foodborne pathogens and *Listeria innocua*. Following incubation for 24 h at 37 °C, the diameter of each clear zone surrounding an 8 mm disc containing 50 µl of supernatant of *L. sakei* NR28, was determined.

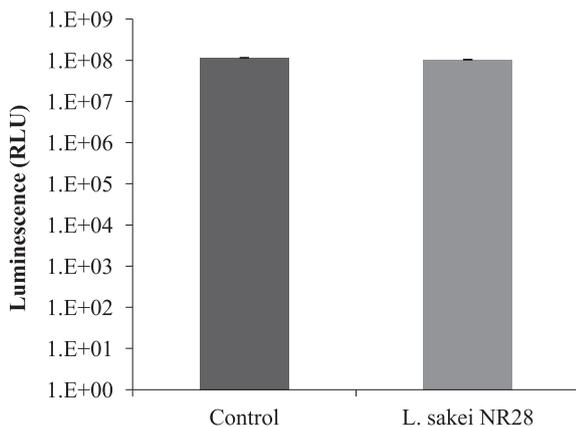
Stains	L.i.	L.m.	B.	S.a.	E.	S.T	P.
Activity	++	++	+	+	-	-	-

+++ : strong positive (diameter >2 mm), ++ (diameter >1.5 mm), + (diameter >1), -: negative; L.i.: *Listeria innocua* KACC 3586; L.m.: *Listeria monocytogenes* KCCM 40307; B.: *Bacillus cereus* ATCC 08715; S.a.: *Staphylococcus aureus* KCCM 11335; E.: *Escherichia coli* ATCC 25922; S.T: *Salmonella* Typhimurium ATCC 14028; P.: *Pseudomonas aeruginosa* ATCC 27853.

adaptation by AI-2 quorum sensing (Buck, Azcarate-Peril, & Kleenhammer, 2009).

Enterohaemorrhagic *E. coli* O157:H7 (EHEC) is an important foodborne pathogen. Its adhesion to epithelial cells and subsequent colonization may result in haemorrhagic colitis and haemolytic-uraemic syndrome (HUS) in the human intestine. This infection process is initiated by the formation of attaching and effacing (AE) lesions on the epithelial cells by type III secretion system (T3SS) and effector proteins (Medellin-Peña, Wang, Johnson, Anand, & Griffiths, 2007). The genes involved in the formation of AE lesion and effector proteins are located on a pathogenicity chromosomal island called the locus of enterocyte effacement (LEE) (Fröhlicher, Krause, Zweifel, Beutin, & Stephan, 2008). According to recent studies, the AI-2 quorum signalling system is also involved in pathogenesis (Bansal, Jesudhasan, Pillai, Wood, & Jayaraman, 2008). Expression of LEE and other virulence genes associated with flagella and motility, can be regulated by quorum sensing (Kendall, Rasko, & Sperandio, 2007). Therefore, the LuxS/AI-2 signalling system plays an important role in EHEC infections of the human intestine, and constitutes an interesting challenge for treatment or prevention of infections caused by EHEC (Kim, Oh, Park, Seo, & Kim, 2008).

Probiotic lactic acid bacteria (LAB) are primary candidates for this approach because of their general acceptability and safety (Medellin-Peña & Griffiths, 2009; Shenderov, 2011), while, commonly, LAB play an important role in the safety and stability of numerous fermented foods. The objective of this study was the development of a new quorum quenching *Lactobacillus* strain. For this purpose we selected the putative probiotic strain *Lactobacillus sakei* NR28, isolated from kimchi in an earlier study (Lee et al.,



**Fig. 1.** Viability of co-cultured EHEC 'wild-type' strain ATCC 43894 using cell viability assay by measuring ATP after 2 h. Initial cell counts of EHEC and *Lactobacillus sakei* NR28 were similar, comprising 10<sup>8</sup> CFU/ml. 1:1 LB and MRS fresh medium were used as control for co-cultivation. The pH was maintained above 6 during cultivation. Data indicate the mean ± SD.

2011), and intended to demonstrate its AI-2 inhibition activity and the AI-2 associated reducing effect on the pathogenicity of EHEC. In addition, using *Citrobacter rodentium*, a new model was applied for mimicking the *in vivo* behaviour of the EHEC strain in a mouse model.

## 2. Materials and methods

### 2.1. Bacterial preparation

#### 2.1.1. Strains

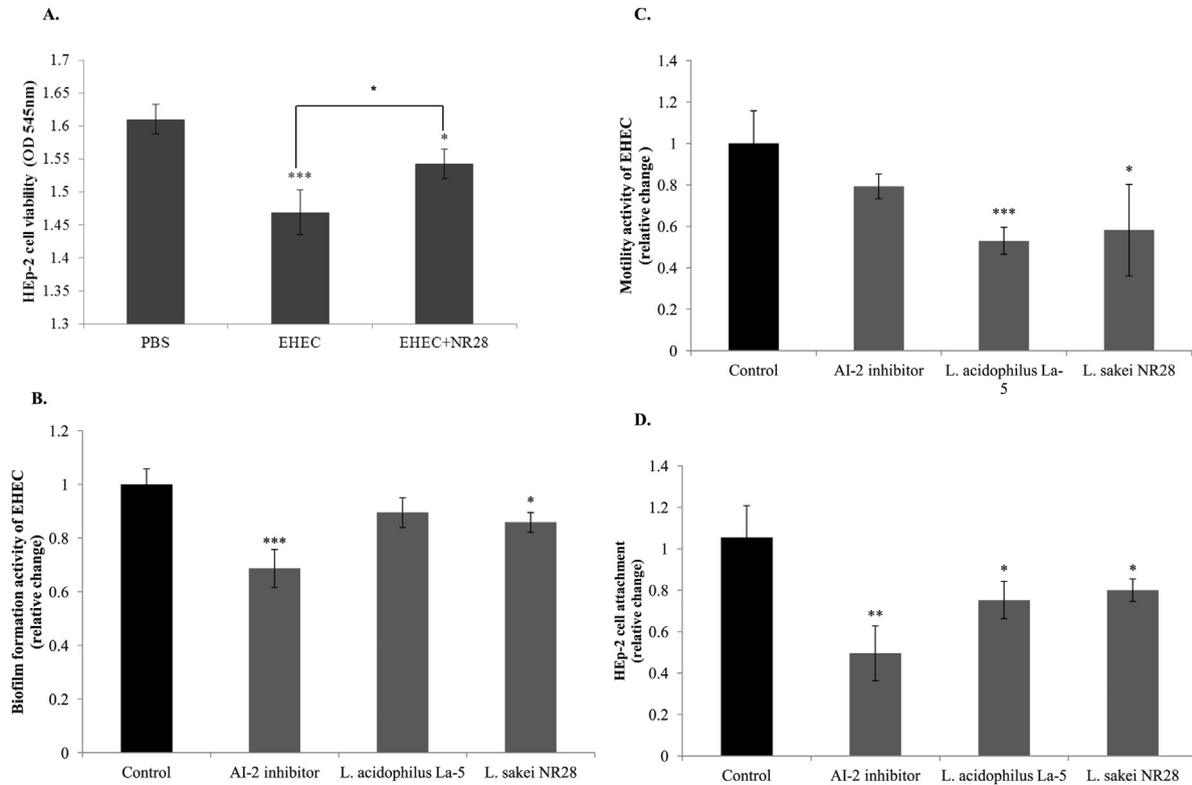
*L. sakei* strain NR28 and *Lactobacillus plantarum* NR74 were isolated from kimchi (Lee et al., 2011). *L. acidophilus* La5 and *Lactobacillus rhamnosus* GG are commercial probiotic strains. *Vibrio harveyi* strains BB152 and BB170 were used for AI-2 bioluminescence assay were obtained from the ATCC (American Type Culture Collection, Manassas, VA, 20110-2209, USA). Enterohaemorrhagic *E. coli* O157:H7 (EHEC) strain ATCC 43894, *E. coli* ATCC 25922, *Listeria innocua* KACC 3586, *L. monocytogenes* KCCM 40307, *Bacillus cereus* ATCC 08715, *S. aureus* KCCM 11335, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, and *C. rodentium* KACC 15163 were used as pathogens. The strains were obtained from the ATCC (American Type Culture Collection, Manassas, VA, 20110-2209, USA), the KCCM (Korean Culture Center of Microorganisms, Seoul, South Korea), and the KACC (Korean Agricultural Culture Collection, Suwon, South Korea), while a *luxS*-deficient mutant of enterohaemorrhagic *E. coli* O157:H7 strain ATCC 43894 (*luxS*-deficient EHEC) was kindly supplied by Prof. Younghoon Kim (Department of Animal Science, Chonbuk National University, Jeonju, South Korea).

#### 2.1.2. Cultivation

*Lactobacillus* strains in this study were incubated at 37 °C for 18 h in 5 ml de Man Rogosa & Sharpe (MRS) medium and sub-cultured twice before use. In the auto-inducer bioassay, *Lactobacillus* strains were grown in modified MRS medium as described by DeKeersmaecker and Vanderleyden (2003). *V. harveyi* strains BB170 and BB152 were grown at 30 °C on an orbital shaker of 125 rpm, either in Marine broth (Difco Laboratories; Becton, Dickinson and Co, USA) or Autoinducer Bioassay (AB) medium (Greenberg, Hastings, & Ulitzur, 1979). Brain-Heart-Infusion (BHI) and Luria-Bertani (LB) media were used for growing EHEC and *C. rodentium* cultures. BacTiter-Glo™ Microbial Cell Viability Assay Kit (Promega, USA) was used for ATP-based bacterial cell number counting. All strains were stored at -80 °C in cultured broth with 20% glycerol added. The stock cultures were propagated twice in each broth for 18 h before each experiment.

#### 2.1.3. Co-culturing

In order to allow the interchange of secreted molecules present in the cultured medium, the bacterial co-culture method by Ruiz, Sanchez, de los Reyes-Gavilan, Gueimonde, and Margolles (2009) was used. The content of each tube was drawn in a sterile syringe and a sterile filter of 0.45 µm pore size (Minisart®) was connected to each syringe. Lactic acid bacteria were grown in MRS broth with 0.1 M MES buffer, and the EHEC strain ATCC 25922 was grown in BHI broth. All bacterial strains were grown overnight and used at an OD<sub>600 nm</sub> of 1.0. They were interconnected by means of single sterile silicon tubing. The media of both syringes were manually mixed every 15 min during a period of 2 h. Cultivation was performed in triplicate.



**Fig. 2.** (A) MTT assay to observe anti-cytotoxicity effect of *Lactobacillus sakei* NR28 on EHEC strain ATCC 43894.  $10^6$  CFU of EHEC/ml were given to a confluent HEP-2 epithelial cell plate. *L. sakei* NR28 supernatant was adjusted to pH 7.0. (B) Effects of supernatant isolated from *L. sakei* NR28 on biofilm formation, (C) motility, and (D) attachment ability of EHEC. Control: fresh MRS broth. Asterisks denote a significant difference (\*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ). Data indicate the mean  $\pm$  SD.

## 2.2. Antimicrobial activity

The paper disc method was used. Each pathogenic test strain was spread on a LB agar plate. A sterile 8 mm disc (Whatman, England) was placed on the agar and 50  $\mu$ l of supernatant of *L. sakei* NR28 was added to each disc. After incubation at 37 °C for 24 h, the diameter of each clear zone was determined. The assay was performed in triplicate.

## 2.3. Cytotoxicity assay

Cytotoxicity of the EHEC 'wild-type' strain ATCC 43894 was measured using an MTT assay. The human epithelial cell line HEP-2

was obtained from the KCLB (Korean Cell Line Bank, Seoul, Korea). HEP-2 was grown on Eagle's Minimum Essential Medium (EMEM, Hyclone, Thermo), supplemented with 10% foetal bovine serum (Hyclone, Thermo), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Hyclone, Thermo) at 37 °C in a 5% CO<sub>2</sub> atmosphere in a tissue culture flask (Corning, USA). Before assay, HEP-2 cells were seeded at  $10^4$  cells per well in 96-well plates and incubated in culture medium for 48 h. Then the cells were treated with  $10^6$  CFU/ml EHEC and 10% fresh MRS medium, and also with EHEC and the 10% *L. sakei* NR28 supernatant, respectively, followed by 4 h incubation. After treatment, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to the cells and incubated for 2 h, and treated with dimethyl sulfoxide. Absorbance was measured at OD<sub>595</sub> nm.

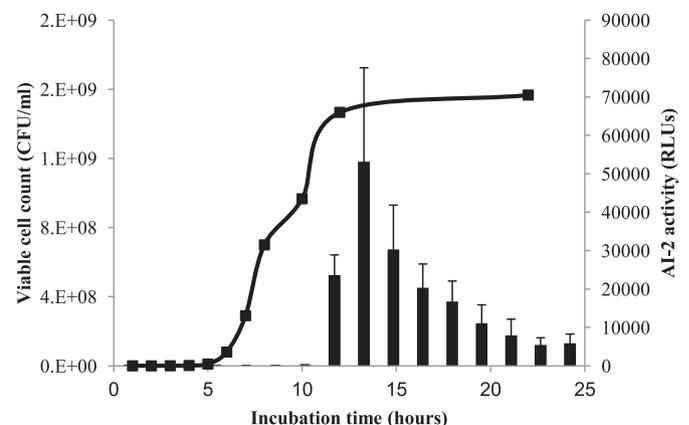
**Table 2**

Inhibitory effects of *Lactobacillus acidophilus* La-5 and *Lactobacillus sakei* NR28 on the gene expression of virulence factors in EHEC. The fold change of virulence transcription was relative to untreated EHEC.

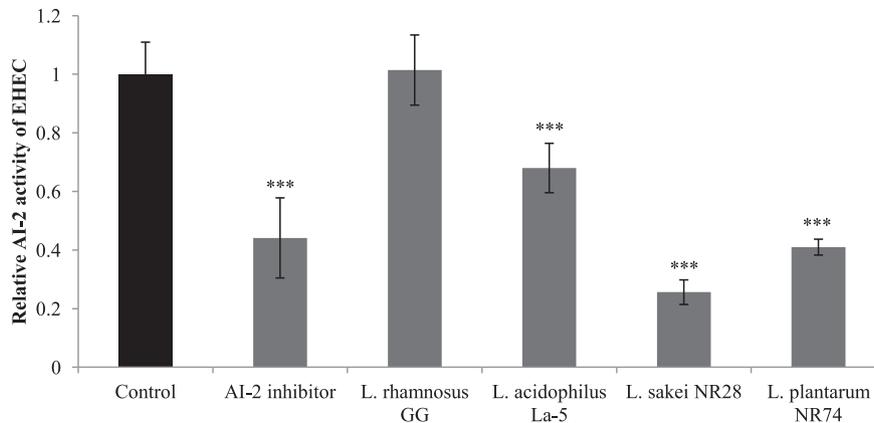
Target gene	Function	Fold change <sup>a</sup>	
		<i>L. acidophilus</i> La-5	<i>L. sakei</i> NR28
<i>luxS</i>	AI-2 synthesis	0.37***	0.32***
<i>qseA</i>	QS <i>E. coli</i> regulator A	0.32***	0.30***
<i>lsrB</i>	AI-2 binding protein	4.33***	5.16***
<i>ler</i>	LEE <i>E. coli</i> regulator	0.09***	0.11***
<i>eae</i>	Intimin	0.26***	0.35***
<i>hlyB</i>	Enterohemolysin B	0.18***	0.17***
<i>tir</i>	Translocated intimin receptor	0.30***	0.24***
<i>flhC</i>	Flagellin	0.94	1.03
<i>espA</i>	EspA protein	0.33***	0.27***
<i>espD</i>	EspD protein	0.38***	0.26***
<i>cysP</i>	Thiosulfate-binding protein	0.18***	0.11***
<i>csgD</i>	promote biofilm formation	0.11***	0.10***

Significance is indicated as follows: \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ .

<sup>a</sup> The expression levels of the studied genes were normalized according to the expression level of the housekeeping gene *tufA*.



**Fig. 3.** AI-2 activity (histogram) and growth curve (■) of *Escherichia coli* O157:H7 'wild-type' strain ATCC 43894 (EHEC) at standard growth conditions in LB broth.



**Fig. 4.** AI-2 activity of *Escherichia coli* O157:H7 'wild-type' strain ATCC 43894 (EHEC) co-cultured with *Lactobacillus* strains *in vitro*. The pH was maintained above 6 during 2 h cultivation by MES buffer. The control comprised a 1:1 MRS-BHI broth mixture with EHEC only. Asterisks denote a significant difference compared with the control (\*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ). Data indicate the mean  $\pm$  SD.

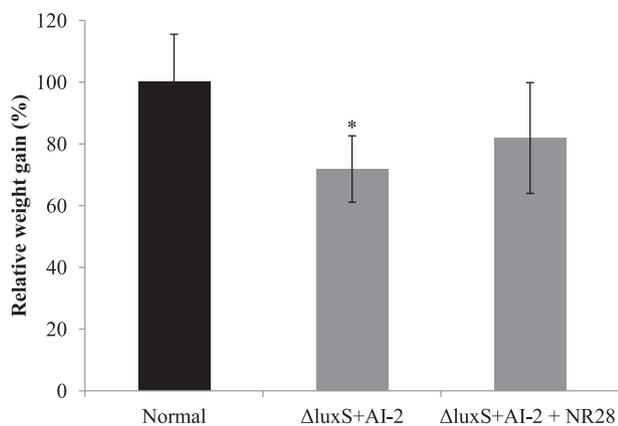
#### 2.4. AI-2 bioluminescence assay

AI-2 activity was measured using a modified bioluminescence assay (Surette & Bassler, 1998). *V. harveyi* BB152, serving as a positive control, and *V. harveyi* BB170, as a reporter, were grown at 30 °C with aeration (125 rpm) for 16 h and adjusted to OD<sub>600 nm</sub> of 0.1 and diluted 1:2000 in AB medium. In a 96-well microtitre white plate (Whatman 7701-3350), cell-free culture medium of either lactobacilli or *V. harveyi* BB152 was added to the diluted *V. harveyi* culture at a final concentration of 10% (v/v). The plate was incubated at 30 °C (125 rpm), and bioluminescence was measured every 15 min after 2 h in a GloMax<sup>®</sup> 96 microplate luminometer (Promega, USA). (Z)-4-Bromo-5-(bromomethylene)-2(5H)-furanone (Sigma–Aldrich) was used as AI-2 inhibitor and (S)-4,5-dihydroxy-2,3-pentanedione (OMM Scientific, Dallas, TX, USA) was applied as purified AI-2 molecule.

#### 2.5. Transcriptional analysis

##### 2.5.1. Bacterial RNA extraction and cDNA synthesis

In order to extract total RNA, bacterial samples were centrifuged (10,000  $\times$  g, 5 min) and re-suspended in 1 ml of



**Fig. 5.** Induction of the pathogenicity of *luxS*-deficient EHEC strain ATCC 43894 by supplementation of the AI-2 signal in an *in vivo* mouse model. Each mouse was infected with the *luxS*-deficient EHEC strain. For supplying the AI-2 molecule, the 'wild type' EHEC strain was incubated in LB broth overnight and 50  $\mu$ l of its supernatant were added. The test group received 50  $\mu$ l of *Lactobacillus sakei* NR28 supernatant additionally. PBS was used as negative control. The experiment was performed during 14 days after infection. (Normal: non-infected mice; ΔluxS: deficient mice; AI-2: signal molecules from supernatant of wild type EHEC strain ATCC 25922; NR28: *L. sakei* NR28). Data indicate the mean  $\pm$  SD (\*,  $P \leq 0.05$ ).

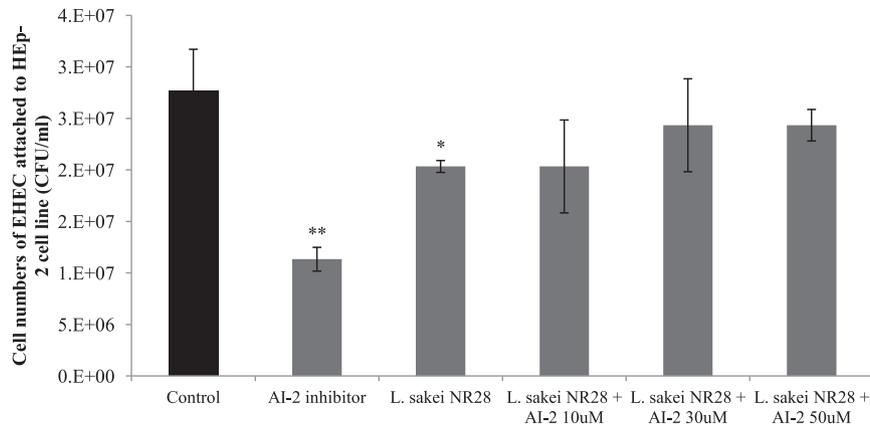
RNAprotect<sup>™</sup> bacterial reagent (Qiagen, Düsseldorf, Germany). The mixed samples were centrifuged at 13,000  $\times$  g for 2 min. After removal of the supernatant, the pellets were immediately stored at –80 °C. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). For the digestion of residual DNA contamination, RNase-free DNase set (Qiagen, Germany) was used. The quantity and quality of the extracted RNA were determined by measuring the absorbance at A<sub>260 nm</sub> and A<sub>280 nm</sub>. The purified RNA was stored at –80 °C. Synthesis of cDNA was conducted using TaqMan Reverse Transcription Reagent (Applied Biosystems, USA) in a DNA amplifier (Applied Biosystems, USA) with the following cycling conditions: 25 °C for 10 min, 48 °C for 30 min, 95 °C for 5 min.

##### 2.5.2. Primers and real-time PCR

Several sets of primers were designed by Bioneer Corporation (Daejeon, South Korea). Gene amplification was carried out on each bacterial cDNA sample using a SYBR<sup>®</sup> Green-based real-time polymerase chain reaction (PCR). All real-time PCR amplifications and analyses were conducted by Accupower<sup>®</sup> qPCR array service (Bioneer, Cat. No. S-6001: Taster kit, using Exicycler<sup>™</sup> 96 Real-Time Quantitative Thermal Block). The entire system and all experiments involved were performed according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines. Data analysis was based on the relative quantitation method, and the fold change of virulence transcription was relative to untreated EHEC. All the data were normalized using the housekeeping *tufA* expression level.

#### 2.6. Measurement of biofilm formation

The biofilm formation protocol was adapted from the method previously described by Kim et al. (2008). Overnight cultures of the EHEC strain were washed twice with PBS and inoculated at 10<sup>6</sup> CFU/ml in fresh 1/10 diluted LB that was supplemented with the 10% (w/v) cell culture supernatants of *L. acidophilus* La-5 and *L. sakei* NR28. After incubation at 30 °C for 48 h without agitation, the microplates (polystyrene and polyvinyl chloride) were thoroughly rinsed twice with sterile distilled water, and a 0.1% (w/v) solution of crystal violet (CV) was added to stain the attached cells. Following staining at room temperature for 20 min, the CV was removed and the wells were gently washed with sterile water three times, and stained dyes were solubilized with 95% ethanol. The absorbance of the solubilized dye was subsequently determined at OD<sub>595 nm</sub>.



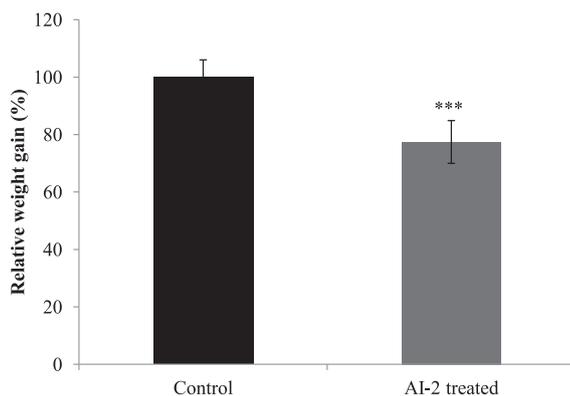
**Fig. 6.** Change in adhesion ability of the EHEC 'wild-type' strain ATCC 43894 when purified AI-2 molecule was added additionally to the HEp-2 cell line. Asterisks denote a significant difference compared with the control (\*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ). Data indicate the mean  $\pm$  SD.

### 2.7. Measurement of motility

Motility of EHEC was assayed as previously described by Bansal et al. (2008). Briefly, an overnight culture of the 'wild-type' EHEC strain was sub-cultured to a turbidity at  $OD_{600\text{ nm}}$  of 0.05 in LB medium and grown to a turbidity of  $OD_{600\text{ nm}}$  1.0 at 37 °C. 30  $\mu$ M AI-2 inhibitor and 10% (w/v) cell culture supernatants of *L. acidophilus* La-5 and *L. sakei* NR28 were added to the motility agar plates (1% tryptone, 0.25% NaCl, and 0.3% agar), respectively, and the grown EHEC cultures were transferred with a sterile needle onto each agar plate. The plates were incubated for 8 h at 30 °C, and the sizes of the halos were measured.

### 2.8. Measurement of adhesion properties

Before assay, HEp-2 cells were incubated to confluence in 24-well plates. EHEC was grown to a turbidity of  $OD_{600\text{ nm}}$  1.0 and washed twice with  $1 \times$  PBS by centrifugation at 16,000 rpm for 5 min, and re-suspend with pre-heated EMEM (37 °C). Confluent HEp-2 cells were washed twice with  $1 \times$  PBS and 1 ml of suspended EHEC was added to each well, followed by 10% cell culture supernatant of the *Lactobacillus* strain, 30  $\mu$ M AI-2 inhibitor, and AI-2



**Fig. 7.** Interference with the anti-pathogenicity effect of *Lactobacillus sakei* NR28 by adding the purified AI-2 molecule *in vivo*. Each mouse infected with the 'wild-type' EHEC strain ATCC 43894 received 50  $\mu$ l of *L. sakei* NR28 supernatant daily (control) and additional treatment with 50  $\mu$ l of 30  $\mu$ M purified AI-2 molecule (AI-2 treated). The experiment was performed during a period of 14 days after infection. Control: NR28 treatment only; AI-2 treated: receiving 30  $\mu$ M of purified AI-2 molecule additionally. Data indicate the mean  $\pm$  SD (\*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ).

molecules (10  $\mu$ M, 30  $\mu$ M, and 50  $\mu$ M). The treated well plates were incubated at 37 °C for 2 h in a 5%  $CO_2$  atmosphere. The cell layers were washed twice with  $1 \times$  PBS and lysed with 100  $\mu$ l of Triton X-100 (0.1% solution) for LB agar plate counting.

### 2.9. In vivo experiments

#### 2.9.1. Animals and feeding-infection

Specific-pathogen-free female ICR mice were obtained at the age of three weeks (Daehan Bio Link Co., Ltd., Eum-sung Gun, Republic of Korea) and used for EHEC and *C. rodentium* feeding-infection experiments with administering of  $5 \times 10^8$  CFU per mouse by oral injection. Each mouse was stabilised for a week before commencing the experiment. All animal experiments were approved by the Institutional Animal Care and Use Committee of Handong Global University, Republic of Korea.

#### 2.9.2. Treatment with purified AI-2 molecule

Each mouse was infected with a single dose of the 'wild-type' EHEC strain ATCC 43894, and also received *per os* 50  $\mu$ l of *L. sakei* NR28 supernatant daily. In addition, one group also received (*per os*) 50  $\mu$ l of the 30  $\mu$ M purified AI-2 molecule. Following infection, the experiment was performed during a period of 14 days.

#### 2.9.3. luxS-deficient EHEC model

Each mouse was infected with the *luxS*-deficient EHEC strain ATCC 43894. For supplying the AI-2 molecule, the 'wild-type' EHEC strain (ATCC 43894) was incubated in LB broth overnight and 50  $\mu$ l of its supernatant were added. The test group received 50  $\mu$ l of *L. sakei* NR28 supernatant additionally. PBS was used as negative control. The experiment was performed during 14 days after infection.

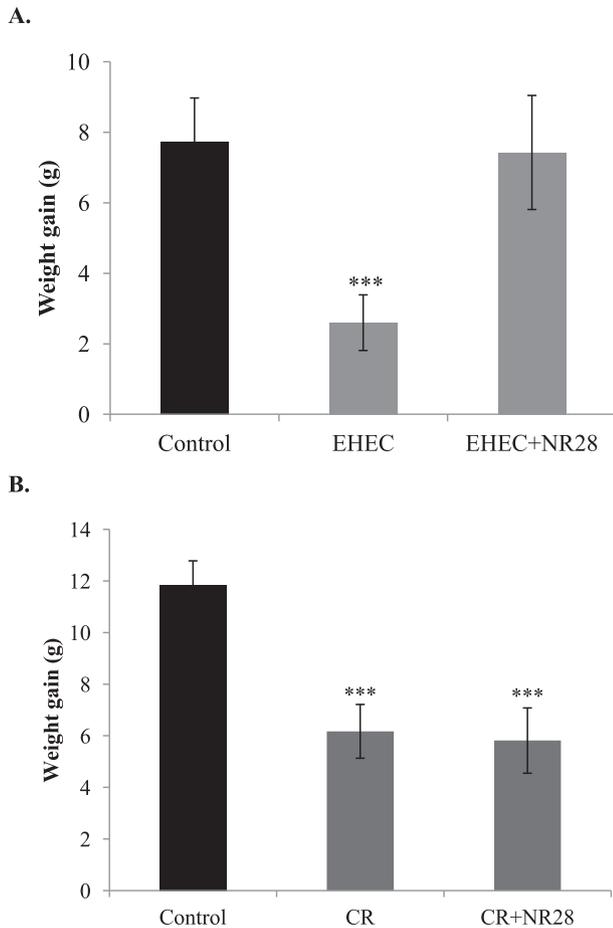
### 2.10. Statistical analysis

The data values were analysed by Student's *t*-test at the  $p < 0.05$  level to evaluate differences among samples of discrete variables.

## 3. Results

### 3.1. Antimicrobial effect

The antimicrobial activity test using the paper disc method (Table 1), showed antimicrobial activity of *L. sakei* NR28 against *L. innocua* KACC 3586, *L. monocytogenes* KCCM 40307, *B. cereus* ATCC



**Fig. 8.** *In vivo* experimental model with four-week old female ICR mice ( $n = 7$ ) for evaluating AI-2 associated anti-pathogenic activity. 50  $\mu$ l of *Lactobacillus sakei* NR28 supernatant was administered every day during 14 days after initial infection with 'wild-type' EHEC strain ATCC 43894. (A) Change of mouse weight gain after infection with EHEC (positive control), and *L. sakei* NR28 treatment. (B) Change of mouse weight gain when infected with *Citrobacter rodentium* (positive control), and an AI-2 independent EHEC mimicking strain, and *L. sakei* NR28 treatment. Negative control: non-infected normal group. Data indicate the mean  $\pm$  SD (\*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ).

08715, and *S. aureus* KCCM 11335, but not against *E. coli* ATCC 25922. Also, in the ATP-based viability assay, *L. sakei* did not affect the viable cell counts of pathogenic *E. coli* (Fig. 1). These results indicate that *L. sakei* strain NR28 did not inhibit the viability of *E. coli* ATCC 25922.

### 3.2. Anti-pathogenic effect

We checked the effects of *L. sakei* NR28 on the practical activity of the virulence factors. In the MTT assay *L. sakei* NR28 inhibited the cytotoxicity of the 'wild-type' EHEC strain ATCC 43894 on HEP-2 cells (Fig. 2A). Biofilm formation (Fig. 2B) and motility (Fig. 2C) of EHEC 'wild-type' strain ATCC 43894 were also significantly reduced by *L. sakei* NR28, and the motility highly significant by *L. acidophilus* La-5. A similar tendency was found for *L. acidophilus* La-5, a commercial probiotic strain for which an AI-2 related anti-pathogenicity effect against *E. coli* O157:H7 has been reported (Medellin-Peña & Griffiths, 2009). Attachment ability is a most important virulence factor of EHEC in the human gastrointestinal tract, and is also affected by AI-2 activity. Thus, we investigated whether the EHEC adhesion ability would be influenced in the presence of *L. sakei* NR28, and found a significant reduction caused

by both this strain and *L. acidophilus* La-5 (Fig. 2D). The relative cell numbers of 'wild-type' EHEC ATCC 43894 on the HEP-2 cell line were reduced 0.8-fold by *L. sakei* NR28. The gene expression of EHEC was also reduced by co-culturing with strain NR28 (Table 2). Genes *cysP* and *csgD*, related to biofilm formation, were strongly inhibited, while LEE regulator gene *ler* and Type-III secretion system related genes were also down-regulated significantly. However, the gene expression for the *fliC* flagella component did not show any change. These results indicate that *L. sakei* NR28 does not inhibit the viability of the pathogenic *E. coli* O157:H7 strain ATCC 43894, although, at the same time, its pathogenicity was clearly reduced. This strongly suggests the selective interference of *L. sakei* NR28 with virulence expression of EHEC.

### 3.3. AI-2 signal inhibition

The production of AI-2 in the 'wild-type' EHEC strain ATCC 43894 under standard growth conditions was measured using an AI-2 bioluminescence assay (Fig. 3). AI-2 activity of EHEC started during mid-exponential phase, while it reached a maximum level in the early stationary growth phase. For the 'wild-type' EHEC at least  $5 \times 10^8$  CFU/ml or higher viable numbers were necessary to achieve a change in expression of the AI-2 signal. The reducing effect on AI-2 activity of the 'wild-type' EHEC strain by co-culturing with *L. sakei* NR28 was indicated by its decreased ATP activity, which was even lower than in presence of 30  $\mu$ M of AI-2 inhibitor (Fig. 4). The results of transcriptional analysis under the same conditions showed that the *luxS* AI-2 synthase and *qseB* quorum regulator genes were reduced by *L. sakei* NR28. However, the *lsrB* AI-2 binding protein gene expression was up-regulated (Table 2), suggesting the down-regulation of AI-2 activity concomitantly with increase in AI-2 sensitivity. In the mouse model, AI-2 transfer by a supernatant of EHEC to the *luxS*-deficient EHEC strain was mitigated when *L. sakei* NR28 was added (Fig. 5), thereby increasing the relative weight gain to an insignificant difference with the control. By comparison, the group receiving only a supernatant of EHEC together with the *luxS*-deficient EHEC strain, showed significant weight loss. This suggests that the putative probiotic strain *L. sakei* NR28 can strongly inhibit the AI-2 signal of EHEC. However, no differences were observed in the behaviour of the experimental groups.

### 3.4. Purified AI-2 molecule treatment

Addition of the purified AI-2 molecule showed that a sufficiently high concentration of the AI-2 signal could restore the adhesion ability of EHEC strain ATCC 25922 in the HEP-2 cell line (Fig. 6). In the mouse model, the reducing effect of *L. sakei* NR28 on adhesion ability of EHEC was also cancelled out by adding purified AI-2 molecules (Fig. 7). This suggests that the reducing influence of *L. sakei* NR28 on the pathogenicity of EHEC is based on *luxS*-related AI-2 signalling activity.

### 3.5. AI-2 independent EHEC mimicking model

Using *C. rodentium* a new model was applied to mimic the *in vivo* behaviour of the EHEC strain in a mouse model. Interestingly, *C. rodentium* has a Type-III secretion system like EHEC (Mundy, MacDonald, Dougan, Frankel, & Wiles, 2005), but AI-2 signalling is not involved in the regulation of virulence factors (Coulthurst et al., 2007). The results showed that in terms of mouse weight gain, the virulence of *C. rodentium* was not affected at all by *L. sakei* NR28 (Fig. 8). This suggests that the anti-pathogenic effect of *L. sakei* NR28 is caused by AI-2 signalling inhibition.

#### 4. Discussion

Producing their virulence factors in the right amount and at the right time constitutes a key factor of pathogens for causing 'effective' damage to the host. The alternative would induce a waste of energy and result in host immune response. Hence, sufficient cell population density is a most important condition required in bacterial pathogenicity (Dangl & Jones, 2001). Actually, several important animal and plant pathogens use quorum sensing to regulate their virulence expression and infectious interaction with the host (de Kievit & Iglewski, 2000; Winzer & Williams, 2001; Zhang & Dong, 2004). Thus, inhibition or regulation of quorum sensing controlling specific virulence gene expression is now being considered as a novel potential target for anti-infective therapy (Bauer & Robinson, 2002; Cámara, Williams, & Hardman, 2002; Finch, Pritchard, Bycroft, Williams, & Stewart, 1998). Several recent studies have provided valuable information on pathogen quorum sensing inhibitors as a new drug target (Smith & Iglewski, 2003; Suga & Smith, 2003). Unlike conventional antibiotics, quorum-sensing inhibitors have less effect on the host microbiota. Also, as blocking of the quorum sensing does not inhibit bacterial growth, the selective resistance mechanisms of the target pathogen were not activated. Moreover, these non-bactericidal anti-pathogenicity effects may decrease the risk of releasing pathogen endotoxins.

EHEC is a highly contagious pathogen due to specific dangerous virulence factors. Due to the release of an endotoxin (Shiga toxin), treatment with an antibiotic has limited potential of success. For this reason, EHEC virulence related quorum-sensing inhibition by probiotics may support antibiotic therapy and may also serve to limit the spread of infections.

In this study we examined the AI-2 associated reducing effect of the putative probiotic strain *L. sakei* NR28 on the pathogenicity of the 'wild-type' EHEC strain ATCC 43894 with the purpose of developing a candidate quorum quenching probiotic strain. The results suggest that strain NR28 had a significant inhibitory effect on the expression of virulence factors in EHEC by AI-2 inhibition (Fig. 2). The inhibition caused a decrease in the expression levels of genes related both to Type-III secretion system, motility, and biofilm formation of EHEC (Table 2). For confirmation and as control, we used purified AI-2 molecules and an AI-2 independent EHEC mimicking strain in an *in vivo* mouse study. Even when the 'wild-type' EHEC was treated with *L. sakei* NR28, the pathogenicity was not reduced when a sufficiently high concentration of the purified AI-2 molecule was either added to the HEp-2 cell line (Fig. 6), or injected additionally in the *in vivo* mouse model (Figs. 7 and 8). *C. rodentium* is a well-reported mimicking strain of EHEC specifically for the mouse model (Mundy et al., 2005). Its pathogenicity is regulated by an AI-1 signalling system (Coulthurst et al., 2007); in our studies, however, *L. sakei* NR28 did not show any effect on AI-1 signalling (data not shown). Therefore, we used *C. rodentium* as a novel model to first evaluate the relationship of AI-2 in the anti-pathogenicity of candidate strains. When the mice were infected with the 'wild-type' EHEC, the group treated with *L. sakei* NR28 showed normal weight gain. However, in the *C. rodentium* infection model, *L. sakei* NR28 treatment did not alleviate the infection. These data thus strongly suggest that the reducing effect of *L. sakei* NR28 on the virulence factors of EHEC is based on *luxS*-related AI-2 signal inhibition. In conclusion, the AI-2 signalling inhibition molecules secreted by the putative probiotic strain *L. sakei* NR28 had reducing effects on the expression of virulence factors in 'wild-type' EHEC strain ATCC 43894.

Kimchi is typically associated with a pool of various lactic acid bacteria (Lee et al., 2005) of which *L. sakei* NR28 may exemplify a link to the beneficial functional properties and safety of this food. In

addition to their "health-promoting" features and 'conventional' antimicrobial activities (production of lactic acid and other antimicrobial metabolites, and competition), some functional strains of lactobacilli from fermented foods such as kimchi show potential for improving food safety thanks to inhibitory effects against pathogens such as EHEC, based on quorum-sensing interference. Prospective clinical applications, however, may open up specific problems. First, the high specificity of quorum-sensing inhibitors seems to be related to a much narrower spectrum of activity. Secondly, as there may still be unknown (or yet unidentified) modes of bacterial communication, the selected inhibitors might cause unexpected inter-species and/or inter-kingdom signalling interaction(s). Thirdly, it seems difficult to develop specific diagnostic systems for investigating availability and dose effect levels (Williams, 2002). Therefore, for the present, it seems feasible to consider the 'assistance' of conventional antibiotics for combating some important pathogens (Ahmed, Petersen, & Scheie, 2007; Xue, Zhao, & Sun, 2013). A first-stage application may aim at improved food safety assurance in a food-processing environment, based on virulence reduction by LAB strains such as *L. sakei* NR28.

More detailed investigations on the 'clinical' appearances of the mice (*in vivo* models) form part of the deepening (and widening) of our current research approach towards clarifying of key mechanisms. Key questions to be addressed include: (a) mode of application; (b) either prevention or treatment of an existing infection; (c) application of living cells or cell-free supernatant; (d) transit/residential time of EHEC in different sections of the GIT; (e) impact of probiotics displaying AI-2 inhibition on physiological activities of the host and/or its microbiota; and (f) correlation of clinical appearances with the load of EHEC.

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