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Effect of CNU091, a Lactic Acid Bacterium, Isolated from Fermented Mustard on Antimicrobial Activity, *in vitro* and *in vivo*

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Abstract The antimicrobial activity of lactic acid bacteria (LAB) isolated from fermented mustard were investigated *in vitro* and *in vivo*. One hundred and fifty nine strains of LAB isolated from traditional Taiwan fermented mustard were evaluated for their adherence ability, acid and bile tolerance and antimicrobial activity *in vitro*. In addition, Wistar rats were orally administered with soy fermented products fermented with CNU091 for their antimicrobial activity *in vivo*. Fecal and fecal of cecum samples were collected to determine number of beneficial bacteria *Bifidobacterium* spp. and harmful bacteria *Clostridium perfringens* by classical plate count and by PCR amplification. Of the strains, CNU091 was acid- and bile-tolerant, and significantly higher adherence to Caco-2 cells compared with the commercial probiotic. CNU091 identified as *Lactobacillus plantarum* with 16S rRNA sequences could inhibit the growth of pathogenic bacteria, including *Escherichia coli*, *Salmonella Enteritidis*, and *Staphylococcus aureus in vitro*. Fecal and fecal of cecum samples were collected to determine number of beneficial bacteria *Bifidobacterium* spp. and harmful bacteria *Clostridium perfringens*, CNU091 had a significant antimicrobial activity compared to the control. The villus height (Vh) / crypt depth (Cd) ratio were increased, which suggested CNU091 modulated intestinal mucin composition. The results implicated CNU091 on antibacterial activity may be regarded as a biological activity and have potential for improving the intestinal health.

Keywords: *antimicrobial potential, fermented mustard, lactic acid bacteria, probiotic effect*

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1. Introduction

Probiotics are bacteria that live in the host human gastrointestinal (GI) tract and have been shown to possess inhibitory activity toward the growth of pathogenic bacteria, resistance to acid and bile salt, adherence to the intestinal epithelial cells and positive effects on the host health [1]. Intake of probiotics stimulates the intestinal microbial balance of the host and reduces the risk of gastrointestinal diseases by improving the growth of beneficial microorganisms and lowering the amount of pathogens [2]. Thus, selection of suitable probiotic candidates is the principal basis for improving the functional properties of probiotic products. Lactic acid bacteria (LAB) are widely utilized in the commercial products of fermented foods and are believed to play important roles in the development and maintenance of health benefit of host. These possible health effects include lowering of cholesterol [3], reducing dental caries [4,5], modulating the immune system [6,7], increasing the antibacterial [5], anticancer and antimutagenic activities and preventing cancer recurrence [7].

LAB have been used in the production of varieties of fermented dairy, vegetables and meat products for many centuries. LAB can produce flavors, odors, textural and nutritional changes in foods [8], and are also known for the inhibitory activity toward the growth of pathogenic bacteria. LAB have the ability to produce antimicrobial compounds such as organic acids (lactic acid, acetic, formic, and others), bacteriocins (nisin, reutericyclin, pediocin, lacticin, and sakacin), reuterin, diacetyl, and/or ethyl alcohol [9,10] to inhibit the growth of intestinal pathogens [11]. Chen *et al.* [12] investigated the in vitro inhibitory properties of the cell free supernatant (CFS) of *Lactobacillus plantarum* against *Streptococcus mutans*. The antibacterial activity of cell-free supernatants (CFS) produced by LAB in vitro against *Escherichia coli*, *Staphylococcus aureus*, *Shigella sonnei*, *Pseudomonas fluorescens* and *Salmonella Typhimuriu*m were found to be effective [8]. Other researchers studied the CFS of

LAB was able to inhibit both Gram positive and Gram negative microorganisms *in vitro* [13].

In Asia, fermented fruits and vegetables products had a long history in human nutrition from ancient ages and were associated with the several social aspects of different communities [3]. Recent studies [3,5,7] were conducted to evaluate traditional fermented vegetables as potential natural sources of probiotic bacteria and select candidates to be used as probiotic starters for the improvement of the traditional fermentation process and the production of new functional food or as a feed additive. Suancai is traditional fermented mustard which is widely used in Taiwan. It is made from green mustard and its production is a spontaneous fermentation process by a mixed microbial population mainly composed of LAB. Previous studies by the authors demonstrated that the lowering cholesterol activity and immunopotentiating effect of LAB isolated from fermented mustard have been reported [3,7]. However, the information related to antimicrobial potential of LAB isolated from traditional Taiwan fermented mustard *in vivo* is limited. Therefore, the aim of this study was to perform *in vitro* and *in vivo* tests to evaluate the antimicrobial potential of LAB strains originating from naturally fermented mustard. The candidate LAB strains that fulfil the established criteria could therefore be potentially used as novel probiotic strains for functional foods.

2. Materials and Methods

2.1. Bacteria Strains and Culture Conditions

A total of 159 LAB strains isolated from suancai, a traditional fermented mustard widely used in Taiwan, were used in this study. In addition, *Lactobacillus casei* Shirota, isolated from a commercial yogurt of Yakult Co., Ltd. (Taipei, Taiwan), as a reference strain was used as the control strain for evaluating the probiotic potential of isolates. The pathogenic strains used as indicator for inhibition study were *Escherichia coli* BCRC 10675, *Salmonella enteritidis* BCRC 10744 and *Staphylococcus aureus* BCRC 13829. The indicator strains were obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). All the isolated LAB strains and indicator bacteria were cultured in MRS broth or Luria broth (*E. coli*, *Salm. enteritidis*), or tryptic soy broth (*Staph. aureus*) and maintained at -80°C in appropriate medium supplemented with 15% (v/v) sterilized glycerol.

2.2. Strains Identification

LAB isolates were characterized by carbohydrate utilization pattern using an API 50 CHL system (bioMérieux Inc., Lyon, France) and identified by 16S rDNA sequence analysis. The preliminary confirmed by API 50 CHL fermentation assays were following the instruction procedure. In 16S rDNA sequence analysis, the PCR primers 27F/1492R designed from the 16S rDNA [14]. The amplification products were purified with DNA purification kit (Viogene BioTek Corp., Taiwan) and sequenced by Center for Genomic Medicine (CGM, National Cheng Kung University, Taiwan). Sequence homologies were examined by comparing the obtained sequence with those in the DNA databases (http//www.ncbi.nlm.nih.gov/BLAST).

2.3. Adhesion Capacity

The ability of the LAB strains to adhere to human epithelial cells was investigated according to the method of Kim *et al.* [15] with minor modifications. Monolayers of Caco-2 intestinal epithelial cells were prepared in Dulbecco's Modified Eagle's medium (DMEM; GIBCO BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% Sodium pyruvate, and 1% penicillin-streptomycin-fungizone mixture in 24-well tissue culture plates at a concentration of 1×10^5 cells/well. Before the adhesion assay, the media in the wells containing a Caco-2 cell monolayer were removed and replaced once with fresh antibiotic-free DMEM. Prior to the adhesion test, the overnight culture of LAB in MRS broth was centrifuged and the cell pellet was washed twice with PBS and resuspended in 1 mL antibiotic-free DMEM. Thereafter, approximately 1×10^7 CFU/ mL of bacteria was added to each well with a total volume of 1 mL and then incubated for 3 hours at 37°C under an atmosphere of 5% (v/v) CO_2. To remove nonattached bacterial cells, the wells were removed with sterile prewarmed PBS. Adherent bacteria were detached by repeatedly pipetting with 1 mL of 0.1% (v/v) Triton X-100 and the cell suspension was plated onto MRS agar and incubated for 48 hours at 37°C to measure the viable cell count. The assay was performed in triplicate for every strain and counts were performed in duplicate.

2.4. Acid and Bile Tolerance

Tolerance to low pH and bile content was assessed as described by Lee *et al.* [6] with slightly modifications. Prior to each of the experiments, all LAB were subcultured twice in MRS broth at 37°C for 24 hours. After cultivation, the bacteria were collected by centrifugation and washed twice with phosphate-buffered saline (PBS, pH 7.2). Then the cell pellets were resuspended in PBS. The ability of the strains to grow at low pH was evaluated in acidified MRS broth (final pH 2.0 or 3.0) containing 1000 U/mL of pepsin (Sigma-Aldrich, USA). The tolerance of the strains to bile was determined in MRS broth containing 0.3% or 0.5% (w/v) oxgall (Sigma-Aldrich, USA). Five milliliters of each type of modified MRS was inoculated with a bacterial suspension to a final cell concentration of approximately 10^9 CFU/mL. Acid tolerance was evaluated by measuring survival after 3 hours of incubation at 37°C. Bile tolerance was evaluated by measuring survival after 24 hours of incubation at 37°C. After cultivation, the bacteria number were estimated.

Survival rate was calculated according to the following equation:

$$Survival\ rate\ (\%) = (log\ CFUN_1 / Log\ CFUN_0) \times 100\%$$

where N_0 represents the total viable count for LAB before treatment and N_1 the total viable count after the treatment at low pH or bile salt, respectively.

2.5. Antimicrobial Activity

Antimicrobial activity was analyzed using the agar well diffusion method described by Vitta *et al.* [16] with some modifications. The pathogenic bacteria used as indicators included Gram-negative and Gram-positive strains, such as *E. coli* BCRC 10675, *Salm. enteritidis* BCRC 10744, and *Staph. aureus* BCRC 13829. Bacteria were grown in Luria broth (LB) or tryptic soy broth (TSB) overnight and diluted to 10^7 CFU/mL and spread on the nutrient agar plates. LAB was incubated in MRS broth medium for 24 hours at 37°C, the spent culture supernatant (SCS) of LAB was collected by centrifugation at 8,500 × g and filtered through a 0.22 μm pore-size sterile filter. Then, 100 μL of filtered LAB-SCS were added to 8 mm diameter wells created on a nutrient agar plate preinoculated with indicator pathogens. The plate was incubated at 37°C overnight and the diameters of the inhibition zones on the agar plate were measured. The inhibitory effect of non-cultured MRS broth was used as a negative control. Each assay was performed in triplicate.

Antagonistic effects of the different conditioning LAB-SCS against pathogenic bacteria were investigated according to the method of Lin *et al.* [2] with some modifications. Pathogens were grown for 18 hours at 37°C in LB broth (*E. coli*, *Salm. enteritidis*), or TSB broth (*Staph. aureus*). Then centrifuged and the supernatant was discarded, and the cell pellet was washed twice with sterile PBS. The spent culture supernatant (SCS) of *Lb. plantarum* CNU091 was collected by centrifugation at 8,500 × g and filtered through a 0.22 μm pore-size sterile filter. The final concentration of pathogens was adjusted to approximately 10^8 CFU/mL in 5 mL of different conditioning solutions, including CNU091-SCS, neutralized CNU091-SCS (adjusted to pH 7.0), heated CNU091-SCS (95°C/30 min) and MRS broth (adjusted to pH 4.0). After co-incubation at 37°C for 4 hours with different conditioning solutions. Colony count assays were determined.

2.6. Preparation of Fermented Soy Milk Culture with Lactic Acid Bacteria

The strain *Lb. plantarum* CNU091 that was selected according to the above described methodology, as well as the reference strain *Lb. casei* Shirota, were further examined for their antimicrobial ability to evaluate the probiotic potential *in vivo*. The culture strain was inoculated 1% (v/v) to soy milk medium, which containing 10 g/L instant non-genetically engineered soy milk powder (Gemfont Corp., Taiwan), 10 g/L soy peptone, 30 g/L glucose, 0.1 g/L NaH_2PO_4, 0.2 g/L $MgSO_4$, 0.1 g/L $MnSO_4$ and 1 g/L sodium citrate. LAB was incubated in soy milk medium for 24 hours at 37°C. After cultivation, the fermented soy milk was analyzed the viable cell numbers and stored at 4°C for feeding rats.

2.7. Animal Grouping and Experimental Design

Male Wistar rats (10 weeks olds) were purchased from the BioLASCO Taiwan Co., Ltd. The rats were maintained in climate-controlled room (at 25°C, 60% humidity) with a 12 hours light/12 hours dark cycle. Food and water were given freely each day. Each rat was visually inspected daily for development of any physical appearance abnormalities during the study period. The body weights were recorded at pre-test and thereafter every two days. The dose of administration was calculated in accordance with Boyd's Formula of body surface area as recommended by the U.S. Food and Drug Administration [1]. In the experiment, A total of thirty-five 10-wk-old Wistar male rats were randomly divided to 5 groups (7 rats/group) including (A) administration of unfermented soy milk medium (control), (B) administration of 1×10^{11} CFU/Kg body weight (bw) of soy milk medium fermented by *Lb. casei* Shirota (positive control), (C) the low dose group (CNU091-L), (D) the mild dose (CNU091-M) and (E) the high dose group (CNU091-H) were orally gavaged with 4×10^9 CFU/Kg bw, 2×10^{10} CFU/Kg bw and 1×10^{11} CFU/Kg bw of soy milk medium fermented by *Lb. plantarum* CNU091, respectively, once a day for 35 consecutive days. The experimental protocol used in this study was reviewed and approved by the Institutional Animal Care and Use Committee of the Chia Nan University of Pharmacy and Science. On day 35, the animals were fasted overnight, and on day 36, the animals were sacrificed using CO_2 asphyxiation. Feces were collected from the cecum and transferred into sampling tubes for intestinal microbiota analyses, DNA extraction and real-time quantitative PCR. The histopathological examinations of rat's jejunum were conducted.

2.8. Analysis of Intestinal Microbiota

Analyses of *Bifidobacterium* spp., and *Clostridium perfringens* in feces were carried out using the method of Tsai *et al.* [17] with slight modifications. A fecal sample (1 g) was suspended in 9 mL of anaerobic diluents, and then a decimal dilution series was prepared. Serial 10-fold dilutions of homogenates were plated on specific media for *Bifidobacterium* spp. (Bifidobacteria iodoacetate media, BIM-25; Creative Media Products, Ltd., Taiwan) or *Cl. perfringens* (Tryptose-sulfite-D-cycloserine agar, TSC agar; Creative Media Products, Ltd., Taiwan) and incubated under anaerobic conditions in an anaerobic chamber for 24 to 48 hours at 37°C. The number of bacteria was expressed as Log_{10} of colony-forming unit (CFU) per gram wet weight of cecal content. The ratio of *Bifidobacteria* spp. was calculated by dividing the number of *Bifidobacteria* spp. by the number of *Bifidobacteria* spp. plus *Cl. perfringens*.

2.9. Real-time Quantitative PCR

Total DNA was extracted from 0.05 g (dry weight) of the cecum content using PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The purity of DNA was estimated on the basis of the OD_{260}/OD_{280} (absorbance at 260 nm/280 nm), which was measured using a spectrophotometer (Hitachi U-2900, E Hong instrument, Co., Ltd, Taiwan).

All the PCR experiments were performed in triplicate using the Applied Biosystems 7000 instrument (Applied

Biosystems Inc., Norwalk, Conn., USA) in 48-well plate format. Total of 20 µL contained 10 µL of 2 × SYBR Green Master Mix (Toyobo, Osaka, Japan), 0.5µL of each primer at final concentration 2.5 µmol /L, and 8µL of template DNA. The amplification reaction consisted of 95°C for 10 min, then 40 cycles of 95°C for 15 sec, then extension at 60°C for 60 sec. *Bifidobacteria* spp. forward primer (F): 5'-GGG TGG TAA TGC CGG ATG-3', reverse primer (R): 5'-TAA GCC ATG GAC TTT CAC ACC-3' [18], *Cl. perfringens* forward primer: 5'-ATG CAA GTC GAG CGA KG-3', reverse primer: 5'-TAT GCG GTA TTA TYC CTT-3' [18]. Quantification was calculated by threshold cycle (C_t) values generated from DNA samples of known concentrations, which was constructed by the reference bacteria.

2.10. Histopathological Examination

Jejunums were removed, cut open lengthwise, and flushed with sterile PBS to remove any traces of digesta. The jejunum was carefully laid out on an ice-cold stainless steel tray and fixed in 10% phosphate-buffered formalin. Then jejunum was embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) for histopathological examination. The histological examination by conventional method was evaluated the morphological changes and the ratios of villi length and crypts depth in the jejunum sections. Sections were studied under light microscope (DIALUX 20 EB, Wetzlar, Germany) at 40 and 400 × magnifications.

2.11. Statistical Analysis

The data are presented as mean ± SD from at least triplicate trials. Data generated from the *in vitro* experiments were analyzed using the one-way ANOVA procedure of SPSS software (IBM Corp., Armonk, NY, USA). The differences among means were detected by the Dunnett's method for multiple comparisons with the control group. Data generated from the *in vivo* experiments were analyzed by one-way ANOVA and the Tukey's HSD method for multiple comparisons with each group. P value below 0.05 ($P < 0.05$) was considered statistically significant for all analyses.

3. Results

3.1. Adhesion Assay

A total of 159 LAB strains were examined for the ability to adhere to Caco-2 cells. Among of them, 9 LAB strains were capable of adhering to Caco-2 cells and showed a statistically significant higher adherence in comparison to the reference strain *Lb. casei* Shirota (Table 1). The LAB isolates were preliminary characterized by carbohydrate utilization pattern using an API 50 CHL system and confirmed by 16S rDNA sequence analysis. The LAB strains including 9 tested strains from fermented mustard identified as *Lactobacillus* spp., *Pediococcus* spp. and *Weissella* spp. as well as a reference strain *Lb. casei* Shirota were further studied (Table 1).

Table 1. Adhesion of the lactic acid bacteria to the Caco-2 cell line

Strain No.	Species	Adhesion of LAB (log CFU/ mL)
CNU018	*Pediococcus acidilactici*	4.93 ± 0.06*
CNU019	*Lactobacillus fermentum*	4.80 ± 0.07*
CNU040	*Lactobacillus plantarum*	4.49 ± 0.13*
CNU042	*Pediococcus pentosaceus*	4.48 ± 0.08*
CNU044	*Pediococcus pentosaceus*	5.74 ± 0.05*
CNU046	*Pediococcus pentosaceus*	4.92 ± 0.05*
CNU091	*Lactobacillus plantarum*	5.44 ± 0.09*
CNU110	*Lactobacillus plantarum*	5.07 ± 0.09*
CNU145	*Weissella cibaria*	4.58 ± 0.19*
Lb. casei Shirota	*Lactobacillus casei*	4.12 ± 0.03

Initial inoculums of each LAB are approximately 1×10^7 CFU/mL ($\log_{10}$ 7.0). Each value in the table represents the mean value ± SD from at least 3 trials. Data were evaluated with one-way ANOVA and compared using Dunnett's test. Values marked with *asterisk* differ significantly from *Lb. casei* Shirota values ($P < 0.05$).

3.2. Acid and Bile Tolerance

To act as a probiotic, it is essential for the bacteria to enable to survive the acidic conditions and resist bile salt in the gastrointestinal (GI) tract and to exert their beneficial effect on the host. Table 2 shows the effect of acid on the viability of tested strains. The relative survival rate of all tested strains were found to be ≤ 20% after 3 hours of exposure to pH 2.0. In contrast, 7 tested strains (CNU018, CNU019, CNU040, CNU042, CNU046, CNU091, and CNU110) showed high resistance to pH 3.0 as well as the reference strain *Lb. casei* Shirota with final populations exceeding 10^8 CFU/mL, and there were no statistically significant differences in viability between 7 tested strains and reference strain *Lb. casei* Shirota at pH 3.0.

The bile is also an important factor which affects the viability of LAB cells in animal intestine. Table 3 shows the effects of different concentrations of bile salt on viability of tested strains. All strains grew well in MRS without bile salt, whereas the majority of tested strains were found to be tolerant to 0.3%-0.5% bile salt even after 24 hours of exposure retaining their viability with minor reduction in viable counts (1-2 Log). Only 2 tested strains (CNU040 and CNU110) demonstrated approximately 3-4 Log reduction after 24 hours of exposure to bile salt.

3.3. Antimicrobial Activity by Agar Well Diffusion Assay

The antimicrobial activity of the tested strains against three different pathogens was determined by agar well diffusion method and the results were shown in Table 4. The pathogenic bacteria used as indicator include Gram-negative bacteria, such as *E. coli*, *Salmonella* spp., and Gram-positive bacteria, such as enterotoxigenic *Staph. aureus*. Reference strain *Lb. casei* Shirota, CNU091 appeared significantly inhibitory to Gram negative (*E. coli* BCRC 10675). Moreover, Table 4 also showed that CNU091 possessed antibacterial ability against all the three indicator strains. The other tested strains showed to varying degrees of strain-specific antibacterial potential against pathogen strains tested.

Table 2. Effects of acidic solution on viability of lactic acid bacteria after 3 h incubation

Strain No.	Final viable counts (log CFU/ mL)			Survival rate (%)	
	0 h	pH 2.0	pH 3.0	pH 2.0	pH 3.0
CNU018	9.53 ± 0.12	2.14 ± 0.01	8.70 ± 0.09	22.5	91.3
CNU019	9.62 ± 0.18	1.79 ± 0.16	8.72 ± 0.17	18.6	90.6
CNU040	9.31 ± 0.21	2.04 ± 0.01	8.05 ± 0.02	21.9	86.5
CNU042	9.38 ± 0.09	1.91 ± 0.04	8.17 ± 0.09	20.4	87.1
CNU044	9.54 ± 0.31	1.83 ± 0.03	7.74 ± 0.09	19.2	81.1*
CNU046	9.58 ± 0.08	1.88 ± 0.04	8.19 ± 0.02	19.6	85.5
CNU091	9.49 ± 0.13	1.94 ± 0.00	8.43 ± 0.11	20.4	88.8
CNU110	9.33 ± 0.10	1.71 ± 0.08	8.32 ± 0.10	18.3	89.2
CNU145	8.68 ± 0.25	1.44 ± 0.04	5.07 ± 0.07	16.6	58.4*
Lb. casei Shirota	9.68 ± 0.01	1.93 ± 0.10	8.61 ± 0.07	19.9	88.9

Each value in the table represents the mean value ± SD from at least 3 trials. Data were evaluated with one-way ANOVA and compared using Dunnett's test. Values marked with *asterisk* differ significantly from *Lb. casei* Shirota values ($P < 0.05$).

Table 3. Effects of bile salt on the viability of lactic acid bacteria after 24 hours incubation

Strain No.	Final viable counts (log CFU/ mL)			Survival rate (%)	
	0 h	0.3%	0.5%	0.3%	0.5%
CNU018	9.59 ± 0.12	8.75 ± 0.01	8.67 ± 0.07	91.2*	90.4*
CNU019	9.64 ± 0.16	8.33 ± 0.02	8.17 ± 0.03	86.4*	84.8*
CNU040	9.51 ± 0.11	6.92 ± 0.07	6.62 ± 0.03	72.8*	69.6*
CNU042	9.40 ± 0.10	8.17 ± 0.18	8.15 ± 0.02	86.9*	86.7*
CNU044	9.14 ± 0.08	5.72 ± 0.09	5.62 ± 0.05	62.6*	61.5*
CNU046	9.26 ± 0.07	8.23 ± 0.02	8.15 ± 0.06	88.9*	88.0*
CNU091	9.64 ± 0.13	8.86 ± 0.05	8.81 ± 0.03	91.9*	91.4*
CNU110	9.44 ± 0.15	6.63 ± 0.08	6.57 ± 0.07	70.2*	69.6*
Lb. casei Shirota	9.62 ± 0.12	7.83 ± 0.04	7.50 ± 0.06	81.4	78.0

Each value in the table represents the mean value ± SD from at least 3 trials. Data were evaluated with one-way ANOVA and compared using Dunnett's test. Values marked with *asterisk* differ significantly from *Lb. casei* Shirota values ($P < 0.05$).

Table 4. Inhibition of pathogenic bacteria by cell-free spent culture supernatant (SCS) of various lactic acid bacteria

Strain No.	Inhibition zone (mm)		
	E. coli	*Salm. enteritidis*	*Staph. aureus*
CNU018	11.9 ± 0.3*	14.3 ± 0.4*	10.4 ± 0.2*
CNU019	13.2 ± 0.3	13.6 ± 0.5	11.9 ± 0.2*
CNU042	12.2 ± 0.6*	12.3 ± 0.4*	11.6 ± 0.2*
CNU046	11.5 ± 0.6*	12.5 ± 0.4*	10.1 ± 0.2*
CNU091	14.7 ± 0.6*	13.9 ± 0.4	12.6 ± 0.2
Lb. casei Shirota	13.5 ± 0.6	13.6 ± 0.4	12.9 ± 0.2
MRS	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0

Inhibition activity on pathogenic bacteria for LAB strain was performed by an agar well diffusion assay. *E. coli* BCRC10675, *Salm. enteritidis* BCRC 10744, and *Staph. aureus* BCRC 13829 were used as indicators. Inhibition activity is presented as diameter (mm) of inhibition zone. Each value in the table represents the mean value ± SD from at least 3 trials. Data were evaluated with one-way ANOVA and compared using Dunnett's test. Values marked with *asterisk* differ significantly from *Lb. casei* Shirota values ($P < 0.05$).

Table 5. Effects of the different conditioning spent culture supernatant (SCS) of *Lb. plantarum* CNU091 on the viabilities of pathogens

SCS samples	Viable counts (log CFU/mL)		
	E. coli BCRC 10675	*Salm. enteritidis* BCRC 10744	*Staph. aureus* BCRC 13829
MRS broth (control)	8.29 ± 0.11	8.86 ± 0.02	8.46 ± 0.06
SCS	2.74 ± 0.21*	0*	4.88 ± 0.07*
Heated SCS	3.50 ± 0.35*	0*	5.42 ± 0.26*
Neutralized SCS	7.91 ± 0.08	7.85 ± 0.03	8.54 ± 0.18

LAB-SCS samples co-incubated with pathogens at 37°C for 4 h. For each pathogen, the inoculum size was 1×10^8 CFU/mL ($\log_{10}$ 8.0). Each value in the table represents the mean value ± SD from at least 3 trials. Data were evaluated with one-way ANOVA and compared using Dunnett's test. Values marked with *asterisk* differ significantly from the control MRS broth ($P < 0.05$).

The antibacterial activity of the cell free spent culture supernatant (SCS) of strain CNU091 against pathogenic bacteria was also examined. The different conditioning solutions, including CNU091-SCS, neutralized CNU091-

SCS (pH 7.0), heated CNU091-SCS (95°C/30 min) and MRS broth (adjusted to pH 4.0) were examined, and *E. coli* BCRC 10675, *Salm. enteritidis* BCRC 10744 and *Staph. aureus* BCRC 13829 were chosen as indicators (Table 5). After 4 hours co-incubated of the CNU091-SCS with pathogens, a dramatic decrease in viability of pathogens were observed. As a control, the MRS broth showed no inhibitory activity. However, the heated CNU091-SCS (95°C/30 min) lost its antagonistic activity insignificantly, indicating that the antibacterial activity produced by *Lb. plantarum* CNU091 was supported by a heat-stable components. In contrast with neutralized CNU091-SCS showed obviously decrease in inhibitory activity. Based on the above results, of the tested strains, strain *Lb. plantarum* CNU091 was found to possess desirable *in vitro* probiotic properties and had good candidates for further investigation with *in vivo* studies to elucidate potential health benefits.

3.4. Analysis of Intestinal Microbiota

In order to verify the direct evidence of *in vitro* antimicrobial activity of CNU091, *in vivo* experiment in rats were conducted. During the course of the experiments, there was no mortality or toxic effect observed in rats after oral administration of CNU091. Table 6 shows the daily weight gain, daily food intake and feed efficiency of rats. The initial body weight (IBW), final body weight (FBW), daily weight gain (DWG), daily food intake (DFI) and feed efficiency of rats, in the experimental groups were comparable with the control groups at the beginning and the end of the experiment (day 35). These were no group-related alternations or statistically differences in IBW, FBW, DWG, DFI, and feed efficiency of rats given *Lb. casei* Shirota or CNU091 at different concentrations, when compared with the control group. These results may indicate that gavage was well tolerated.

Table 7 shows the number of fecal or cecum microbiota in the rats fed with different concentrations of CNU091. These were no significant differences in the number of *Bifidobacteria* spp. (nB) and the number of *Cl. perfringens* (nCl) among all the samples. However, the ratio of nB/(nB+nCl) of the fecal or fecal of cecum of rats fed with CNU091 at high concentration was higher than that of the control.

Table 8 shows the relative DNA amounts of *Bifidobacteria* spp. and *Cl. perfringens* in fecal of rats' cecum. The C_t value of *Bifidobacteria* spp. (C_tB) and *Cl. perfringens* (C_tCl) from rats fed with CNU091 at different concentrations and at *Lb. casei* Shirota did not differ from each other. The ratio of C_tB/C_tCl of rats fed with CNU091 at different concentrations was lower than the control, which may indicate that CNU091 can improve balance of beneficial bacteria and harmful bacteria.

Table 6. Daily weight gain, daily food intake and feed efficiency of rats

Groups	Initial body wt. (IBW, g)	Final body wt. (FBW, g)	Daily wt. gain (DWG, g day^{-1})	Daily food intake (DFI, g day^{-1})	Feed efficiency
Control	442.71 ± 17.07	537.43 ± 22.49	2.25 ± 0.23	33.10 ± 3.38	6.83 ± 0.52
Lb. casei Shirota	411.71 ± 27.44	500.57 ± 36.53	2.11 ± 0.46	31.08 ± 0.88	6.80 ± 1.41
CNU091-L	445.71 ± 34.34	525.00 ± 44.90	1.89 ± 0.38	31.41 ± 2.65	6.00 ± 1.05
CNU091-M	437.57 ± 33.97	521.43 ± 40.95	2.00 ± 0.28	30.93 ± 2.92	6.46 ± 0.79
CNU091-H	449.57 ± 21.16	539.00 ± 21.83	2.13 ± 0.22	29.88 ± 2.14	7.17 ± 0.94

Each value in the table represents the mean value ± SD for seven rats per group. Values in the same column with different superscript letter $^{(a, b)}$ are significantly different at $P < 0.05$ analyzed by Tukey's HSD test. Feed efficiency = (daily weight gain/daily food intake) × 100%.

Table 7. Effect of the number of fecal or cecum microbiota in the rats fed with different concentrations of *Lb. plantarum* CNU091

Groups	fecal			fecal of cecum		
	Bifidobacteria (nB) (Log cfu g^{-1})	*Cl. perfringens* (nCl) (Log cfu g^{-1})	nB/(nB+nCl) (%)	*Bifidobacteria* (nB) (Log cfu g^{-1})	*Cl. perfringens* (nCl) (Log cfu g^{-1})	nB/(nB+nCl) (%)
Control	7.45 ± 0.69	7.41 ± 0.66	52.35 ^{b}	6.94 ± 0.32	6.94 ± 0.30	50.19 ^{b}
Lb. casei Shirota	7.28 ± 0.64	7.20 ± 0.65	54.30 ^{b}	6.75 ± 0.33	6.69 ± 0.30	53.16 ^{ab}
CNU091-L	7.59 ± 0.43	7.38 ± 0.45	61.24 ^{ab}	7.50 ± 0.75	7.21 ± 0.70	64.84 ^{ab}
CNU091-M	7.58 ± 0.18	7.30 ± 0.33	63.75 ^{ab}	7.41 ± 0.62	7.11 ± 0.38	64.27 ^{ab}
CNU091-H	7.65 ± 0.32	7.35 ± 0.40	66.39 ^{a}	7.42 ± 0.48	7.01 ± 0.54	70.57 ^{a}

Values are means ± SD for seven rats per group. Values in the same column with different superscript letter $^{(a,b)}$ are significantly different at $P < 0.05$ analyzed by Tukey's HSD test.

Table 8. Relative DNA amounts of *Bifidobacterium* spp. and *Cl. perfringens* in fecal samples of rats' cecum

Groups	C_t value		Ratio of C_tB/C_tCl
	Bifidobacteria (C_tB)	*Cl. Perfringens* (C_tCl)	
Control	34.10 ± 0.86	30.03 ± 4.53	121.51 ± 3.41 ^{b}
Lb. casei Shirota	34.20 ± 1.21	27.40 ± 5.07	119.63 ± 10.14 ^{ab}
CNU091-L	34.61 ± 1.93	33.08 ± 4.10	110.53 ± 6.22 ^{a}
CNU091-M	34.61 ± 1.72	32.31 ± 4.57	111.87 ± 6.81 ^{a}
CNU091-H	33.65 ± 1.11	26.94 ± 2.41	111.86 ± 5.06 ^{a}

Values are means ± SD for seven rats per group. Values in the same column with different superscript letter $^{(a,b)}$ are significantly different at $p < 0.05$ analyzed by Tukey's HSD test.

Table 9. The ratios of villi length and crypts depth in the jejunum sections

Groups	Villus height (V_h) (mm)	Crypt depth (C_d) (mm)	Ratio of V_h/C_d
Control	0.60 ± 0.15^{c}	0.23 ± 0.03^{b}	2.70 ± 0.68^{b}
Lb. casei Shirota	0.70 ± 0.13^{b}	0.29 ± 0.02^{ab}	2.47 ± 0.59^{b}
CNU091-L	0.95 ± 0.24^{a}	0.37 ± 0.19^{a}	3.00 ± 0.65^{ab}
CNU091-M	0.90 ± 0.15^{ab}	0.30 ± 0.72^{ab}	3.14 ± 0.87^{ab}
CNU091-H	0.85 ± 0.12^{ab}	0.23 ± 0.46^{b}	3.83 ± 0.49^{a}

Values are means ± SD for seven rats per group. Values in the same column with different superscript letter $^{(a,b)}$ are significantly different at $P < 0.05$ analyzed by Tukey's HSD test.

3.5. Histopathological Examination of Rats' Jejunum

Table 9 shows villus height (V_h) and crypt depth (C_d) of rats fed with CNU091. CNU091 at different concentrations and *Lb. casei* Shirota showed significant higher in V_h compared to the control group. However, no statistically significant difference (p>0.05) was observed in V_h of CNU091 at different concentrations. It was significant differences (p>0.05) in C_d of rats given CNU091 at low concentration, when compared to the control group. However, the ratio of V_h/C_d in rats' jejunum fed with CNU091 at high concentration was higher than that of control.

4. Discussion

Probiotics are believed to temporarily colonize the intestine by adhering to intestinal surface. Therefore, the adhesive ability of bacteria to intestinal cells is considered as one of the selection criteria for probiotic strains [2]. In addition, the Caco-2 cell line has been widely used as an *in vitro* model for intestinal epithelium and the cell line has been used to screen for adhesive strains [19]. In this study, the adhesion of CNU091 was significantly better than the adhesion of *Lb. casei* Shirota. Many studies noted that retention in the intestinal tract is dependent on absorption of the bacteria to epithelial surfaces [20]. Strain CNU091 has marked ability to absorb to intestinal surfaces. Many studies reported that the composition of the human intestinal microbiota play an important role in health and disease. For example, the presence of a large of lactobacilli and bifidobacteria has been considered essential to promote intestinal health and to strengthen the local immune response [21]. The benefits of probiotic bacteria are mainly dependent on their ability to survive, colonize and multiply in the host [22]. However, there is a considerable loss in viability of probiotic bacteria when they encounter the very acidic conditions of the stomach and high bile concentration in the small intestine [22]. According to the results from Table 2, a reduction in total colony forming units was found in the selected strain CNU091 after incubation at pH 2.0 for 3 hours. In contrast, the selected strain CNU091 decreased by 1 Log cycles and exhibited the survival ratio of 88.8% after incubation at pH 3.0 for 3 hours. Clearly, CNU091 demonstrated a significant acid tolerance, which indicated that CNU091 showed marginal loss in viable cell concentration over 3 hours period of incubation in stimulated gastric acid. The finding is meaningful since strain CNU091 resistant to acidity is important for the manufacture of fermented foods and provides health as well [3].

Acid tolerance and bile tolerance are important probiotic characteristics. Therefore, we next conducted whether selected strains are bile tolerance. The bile salt concentration in the gut ranges from 1.5% to 2% in the first hour of digestion, while levels decrease afterwards to around 0.3% w/v [23]. Therefore, to stimulate the small intestine conditions, a broader range of bile concentrations, 0.3% and 0.5% of bile salt concentrations, were tested in the present study. From Table 3, all selected strains showed good growth in MRS broth without bile salt, however, the majority of tested strains were found to be tolerant to 0.3%-0.5% bile salt even after 24 hours of exposure retaining their viability with minor reduction in viable counts (1-2 Log). Of special note that strain CNU091 showed higher survival in the presence of bile salt than *Lb. casei* Shirota. Bile salt are toxic for living cells, since they disorganize the lipid bilayer structure of the cellular membranes [24]. According to the data obtained from Table 3, it seems that the increase in bile salt resistance induced in strain CNU091 could conferred on this microorganism a greater capacity to tolerate the toxic action of bile salts, and thereby leads to better adapted to growing and surviving in the intestine [24].

In Taiwan, *Staph. aureus* and *Salmonella* are considered the second and third common bacterial pathogen causing outbreaks of food poisoning, after *Vibrio parahaemolyticus*. In addition, *E. coli* playing an important role in maintaining normal gut physiology, is a kind of opportunistic pathogen and may cause some infections, such as peritonitis, cystitis etc [25]. To control the development of these pathogens in foods, in addition to traditional chemical and physical preservatives, several bacteriocins produced by lactic acid bacteria have been used with varying degree of success [5]. Thus, when the cultures of *E. coli*, *Salm. enteritidis* and *Staph. aureus* were co-incubated with the different conditioning LAB-SCS of *Lb. plantarum* CNU091, viable counts were markedly and significantly reduced after 4 hours incubation except in neutralized SCS-CNU091. Many studies have been reported that antimicrobial substances produced by lactic acid bacteria include lactic acid, acetic acid, hydrogen peroxide, diacetyl, carbon oxide, bacteriocins, antibiotics [26]. Lin *et al.* [27] also indicated that cell cultures of LAP5 strain were neutralized to pH 7.0, the antagonistic effects of LAP5 against the *Salmonella* growth showed no inhibitory activity. It recommended that the antimicrobial activity was heat stable and required an acidic environment to optimally develop its activity. Thus, pH, organic acids or bacteriocin in the CNU091-SCS which act at low pH conditions may play an important role in antagonistic effect.

In order to verify the direct evidence of *in vitro* antimicrobial activity of CNU091, *in vivo* experiment in

rats were conducted. The balance between pathogenic and non-pathogenic members of the microbiota has been suggested to influence systemic and gastrointestinal health [28]. *Bifidobacterium* spp. and *Cl. perfringens* are the most important bacteria in the gastrointestinal tract, and they are considered as beneficial and harmful bacteria, respectively [29]. Therefore, *Cl. perfringens* and *Bifidobacteria* spp. were selected as reference bacteria in this *in vivo* experiment. Because the changes of fecal microbiota can reflect the change of intestinal microbiota, in this study therefore fecal samples of rats were used to evaluate the effects of CNU091 on intestinal microbiota [29]. In the beginning, we removed spent cell supernatants (SCS) and only used CNU 91 cells to feed rats, compared with the control group, there was no significant difference in cell members of the two bacteria in all group, as well as no significant changes of the ratio of *Bifidobacteria*/(*Bifidobacteria* + *Cl. perfringens*) in fecal were observed (data not shown). There has been great progress in the development of the so called probiotic products, which are food supplements containing live micro-organisms that confer a health benefit on the host when administered in adequate amounts. Prebiotics are non-viable food components that confer benefits to host health associated with the modulation of their intestinal microbiota. The positive influence of prebiotic substances, in intestinal flora has been tested in several studies, where the utilization of probiotic species in combination with prebiotic substances provides a combined effect called ‘‘synbiotic”[30]. Soy products have an excellent nutritional status based on their high protein, essential vitamins, minerals and phytoestrogens content [30]. In this study, CNU091 and soy fermented products containing prebiotics and probiotics (synbiotic food) after fermentation by CNU091 were used to feed rats with different concentrations. According to the data in Table 7, compared with the control group, there was also no significant difference of the two bacteria in all group, however, significant changes of the ratio of *Bifidobacteria*/(*Bifidobacteria* + *Cl. perfringens*) in fecal and in fecal of cecum were observed in CNU091 at high concentration group at day 35, respectively. Obviously, the populations of both *Bifidobacteria* spp. and *Cl. perfringens* were not altered significantly by CNU091 feeding, whereas fermented liquor feeding resulted in greater ratio of both microbes populations than in the control, suggesting that soy fermented culture containing SCS and CNU091 cells do play a valuable role in improving intestinal health.

RT-PCR analysis has been proven to be a simple and reliable technique to quantity microorganisms because the changes in microbial community composition can be determined by RT-PCR [29]. Therefore, RT-PCR was employed to quantitative the bacteria in our study. The data from the *in vivo* experiment demonstrated that there was no significant difference in the genome copies of *Bifidobacterium* spp. and *Cl. pefringens* in fecal of rat cecum in all groups, however, the ratio of *Bifidobacterium*/*Cl. perfringens* (C_tB/C_tCl) in genome copies from fecal of cecum of rats fed with CNU091 at different concentration was lower than the control group. This indicated that CNU091 had a positive effect on caecal microbiota that may be related to the health of the host [25].

Generally, V_h and V_h/C_d ratio may represent an indicator of the digestive capacity of the small intestine and higher values are concomitant with improved digestion and adsorption [31]. According to the results from Table 9, higher V_h was seen in the small intestine of rats fed with CNU091 at different concentrations. Moreover, higher V_h/C_d was seen in the rats fed with CNU091 at high concentration, compared to the control. Pelicano *et al.* [32] noted that the higher villi may have resulted from the action of organic acid (those added to the diet with the probiotics in conjunction with the acids produced by the microbiota), which contribute to a more effective pH reduction in the intestine and, consequently, reduced colonization of the intestine by enteropathogenic microorganisms. In addition, maximum absorption and digestion capacity is given by a large luminal area with high villi and mature enterocytes [32]. Therefore, beneficial effects were observed in the intestinal mucosa with the use of probiotics, CNU091. This finding provides indications that CNU091 has potential to be beneficial for improving the intestinal function.

5. Conclusion

The present study provides new findings about the influence of CNU091, isolated from fermented mustard, on intestinal tract. Strain CNU091 with remarkable antimicrobial activity is acid and bile salt tolerance. In particular, strain CNU091 demonstrated strong adherence to Caco-2 cells *in vitro*. Furthermore, soy fermented culture containing SCS (spent culture supernatant) and CNU091 cells have positive effects on the populations of *Bifidobacterium* spp. and *Cl. perfringens in vivo*. The results of this study demonstrated that supplementation with strain CNU091 results in a significant probiotic effect, which has the potential to be beneficial for improving the intestinal ecosystem function. However, further scientific clinical trials are necessary, to prove the probiotic effect of CNU091.

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Disclosure

The authors declare no relevant conflicts of interest to report.

List of Abbreviations

Cd, crypt depth; CFU, colony forming unit; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GI, gastrointestinal; nB, number of Bifidobacteria spp; Vh, villus height

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