Food Control 69 (2016) 5–12

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Evaluation of functional properties of lactobacilli isolated from Korean white kimchi

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ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form
16 April 2016
Accepted 20 April 2016
Available online 22 April 2016

Keywords:
White kimchi
Lactic acid bacteria
Lactobacillus
L. sakei
L. plantarum
Safety
Functionality

ABSTRACT

Kimchi, probably Korea's most famous traditional fermented food, is well known for its beneficial properties. Among several hundred different types of kimchi in Korea, white (*baek*) kimchi is prepared without chilli and is widely appreciated also by non-Koreans because of its unique mild flavour. In an approach to identify the bacteriological basis for proposed health benefits, we isolated 11 *Lactobacillus* strains from six samples of white kimchi, and investigated their safety and functional features. These strains represented the species *Lactobacillus brevis, Lactobacillus plantarum* and *Lactobacillus sakei* that dominated the populations within a range of 3×10^6 to 4×10^8 CFU/mL. Following safety assessment based on antibiotic resistance and biogenic amine production, 7 different strains were selected for further studies including evaluation of their adaptation to cabbage juice and resistance to phenol. Growth in and adaptation to the cabbage juice was favourably influenced by addition of 2% salt. Final selection was based on *in vitro* passage of simulated stomach duodenum conditions (SSDP model). The strains *L. plantarum* HAC01 and *L. sakei* HAC10 were administered to a diet-induced obese (DIO) mouse model receiving a high-fat (HF) diet to assess their functionality *in-vivo*. Animal groups receiving the viable strains showed significantly lower body weight and total weight gain during 8 weeks compared to the high-fat control group. This study provides preliminary information on the use of *in-vitro* and *in-vivo* features for safety and functionality evaluation of *Lactobacillus* strains from white kimchi. These "first-level" criteria for strain selection may serve as model, thereby facilitating potentially new probiotic developments.

1. Introduction

Fermentation is one of the most widely used practices of preserving staple foods around the world. Over the centuries diverse developments in food fermentations were driven by the need for safe and wholesome foods, environmental factors, and the availability of raw materials and condiments, and, in addition, were influenced by consumer preferences and cultural traditions. Fermentation is not only a means of increasing the shelf life of a food raw material, but is also known to influence quality and functionality of foods in a positive way, e.g., by improving taste and flavour, and beneficially impacting host health. Positive perceptions of microbes are thus associated with desired changes in the food raw material during fermentation. Traditionally, fermented foods have been valued by many cultures for their health benefits and even therapeutic properties (Holzapfel, 2002; Mathara, Schillinger, Kutima, Mbugua, & Holzapfel, 2004). Beneficial health effects of fermented foods are closely related to specific bacteria, in particular lactic acid bacteria (LAB). Studying the interactions of these bacteria within the gastro-intestinal ecosystem has become a major challenge towards clarifying the complex mechanisms basic to the claimed health effects. This area represents a challenging and exciting field of multidisciplinary research, both for gastroenterologists, molecular biologists, microbiologists, food scientists and human physiologists. In spite of developments in food processing and preservation techniques, allowing us to enjoy fresh and safe dishes daily, an increasing number of scientific publications are

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http://dx.doi.org/10.1016/j.foodcont.2016.04.037
0956-7135/

revisiting benefits of functional microorganisms involved in traditional fermentation. Thus far, fermented dairy products such as yogurt have been considered both as potential source of beneficial (probiotic) bacterial strains and an ideal matrix for delivering such functional strains (Rivera-Espinoza & Gallardo-Navarro, 2010). However, part of the focus has now been shifted to a range of non-dairy fermented fruits and vegetables, typical of Asia (Swain, Anandharaj, Ray, & Parveen Rani, 2014) and fermented plant raw materials, in particular cereals, of Africa (Franz et al., 2014) and Europe (Todorov et al., 2008) as ecosystems of potentially beneficial stains.

Beneficial microorganisms can be grouped according to definitions suggested by the *European Food and Feed Cultures Association* (EFFCA, 2015). Microbial Food Cultures (MFC) are defined as preparations or formulations "consisting of concentrates of one or more microbial species and/or strains including unavoidable media components carried over from the fermentation and components, which are necessary for their survival, storage, standardisation and to facilitate their application in the food production process". Starter cultures comprise "MFC preparations used as food ingredients in one or more stages in the food manufacturing process, which develop the desired metabolic activity during the fermentation or ripening process. They contribute to one or multiple unique properties of food stuff especially in regard to taste, flavour, colour, texture, safety, preservation, nutritional value, wholesomeness and/or health benefits" (EFFCA, 2015; Herody, Soyeux, Hansen, & Gillies, 2010). Strains exerting health benefits are collectively grouped as probiotic cultures based on a "consensus definition" by WHO/FAO, (2001) as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host".

Kimchi is probably the most famous traditional fermented food of Korea. It is prepared mainly with Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), but other raw materials such as radish and leek are also being used in conjunction with various condiments such as garlic, ginger and red pepper (chilli), resulting in several hundreds of different recipes. The type and combination of raw materials used may decisively influence the diversity and domination of LAB involved in the fermentation. Among the different types of kimchi "*baek kimchi*" is rather special because of its white colour and mildness, contrasting most types of kimchi characterised by a red colour by addition of chilli, the most common condiment. *Leuconostoc* spp. typically dominate the early phases of kimchi fermentation, and are soon succeeded by *Lactobacillus* spp., bringing the total population up to $>10^8$ CFU/mL after 6 days at 15 °C, and a pH around 4.2, with a subsequent steady decrease of the population (Kim, Ban, Beuchat, Kim, & Ryu, 2012).

A clinical study has shown that consumption of fermented kimchi for three months resulted in reduced body fat mass in obese and overweight patients compared to the group consuming fresh (unfermented) kimchi. Moreover, other health indicators such as blood pressure, glucose, insulin and cholesterol levels were also significantly reduced in the group consuming fermented kimchi (Kim et al., 2011). When comparing fresh (unfermented) kimchi with the fermented product, the results imply that lactic acid bacteria (LAB), representing the dominating microorganisms in the fermented product, play a beneficial role towards improving the obese-related status of the host. Several other studies have shown an effect of LAB strains against metabolic disease. However, results are still controversial and the mechanisms for explaining the effect still require further in depth studies (Kadooka et al., 2010; Lee, Jenner, Low, & Lee, 2006; Wang et al., 2015). We therefore considered it a major challenge to link postulated anti-obesity properties of kimchi with specific LAB strains.

The purpose of this study was to isolate LAB strains typical of Korean white (baek) kimchi and to select potentially probiotic strains based on their functional properties. Our focus has been on strains of *Lactobacillus* spp., representing the major putatively probiotic group within the LAB. In this study we isolated several *Lactobacillus* strains and, after confirming their safety and identity, evaluated their functional characteristics under both in-vitro and in-vivo conditions with the view on their potential application as beneficial probiotic strains.

2. Materials and methods

2.1. Isolation and identification of LAB strains from white kimchi

Samples of white kimchi were obtained in a state of active fermentation from local Korean markets and restaurants and analysed for the viable bacterial population. Not knowing the exact former history, fermentation was continued for a period of 10–15 days at 4 °C after purchase. The pH of each sample was measured using an Orion 2-Star pH meter (Thermo Scientific, USA) directly before plating of a sample. Each kimchi sample was blended with 90 mL of NaCl (0.85% m/v) in a sterile plastic bag for 5 min at 200 rpm using the Stomacher®400 Circulator (Seward, UK) and, after serial dilution in 0.85% NaCl, spread plated onto an MRS agar plate (5.5% of Lactobacillus MRS broth, BD Difco, USA, and 1.5% of Bacteriological Agar, Affymetrix, USA). After incubation at 37 °C for 24–48 h, colonies from the highest dilutions (10^{-6} and 10^{-7}) were selected for isolation and purification. Comparative colony morphology was used as a first step for quantifying presumptive strain diversity on a plate, and was followed by microscopy (Imager.A2, ZEISS, Germany) for determining the cell morphology, with rod-shapes varying from short rods (single and in pairs) with rounded ends (presumptively *Lactobacillus brevis*), to "plump" short to medium short rods (presumptively *Lactobacillus plantarum*), to coccoid rods (presumptively *Lactobacillus sakei*). Catalase activity was determined from colony growth on MRS agar (BD Difco, USA) at 37 °C for 24–48 h by using 3% H_2O_2. Catalase negative strains were identified by bi-directional 16S rDNA sequencing performed by Solgent (Korea), and further analysed using BLAST database with a sequence-matching program.

2.2. Bile salt deconjugation

To assess bile salt hydrolytic (BSH) activity of each strain, the BSH screening medium of Dashkevicz and Feighner (1989) was used and 10 μL of each strain were inoculated onto a 5 mm paper disc on the plate. A noticeable precipitation zone after 24–48 h incubation at 37 °C was considered as indication of bile salt deconjugation.

2.3. Haemolysis and gelatine hydrolysis

Bacterial strains grown at 37 °C for 16–18 h were used for both experiments. Ability to produce hemolysins was determined by streaking each strain onto a blood agar plate containing 5% of sheep blood (Hanil Komed, South Korea), followed by incubation at 37 °C for 24–48 h. The gelatine hydrolysis test was performed in Nutrient Gelatine medium, which contained 5 g/L of peptone, 3 g/L of beef extract and 120 g/L of gelatine. The medium was distributed in test tubes and strains were stab-inoculated into it. After incubation at 37 °C for 24, 48 and 72 h, the media were placed in ice for 15 min and then checked for liquefaction (Dela Cruz & Torres, 2013).

2.4. Biogenic amine formation

Strains grown in MRS broth at 37 °C for 16 h were cultured in a

special medium according to Bover-Cid and Holzapfel (1999) for 48 h at 37 °C. Sterile MRS broth was used as a negative control. Using the precursor amino acids tyrosine, histidine, ornithine, and lysine, respectively, four variations of this medium were prepared to detect production of the biogenic amines tyramine, histamine, putrescine and cadaverine. Biogenic amine production was determined by colour change of the pH indicator bromocresol purple in the medium. Only strains showing a negative reaction were subjected to further selection tests.

2.5. Determination of antibiotic resistance

Each antibiotic was serially diluted (1:2) and mixed into MRS agar medium at concentrations of 0.025–64 μg/mL. Antibiotics used for this experiment were erythromycin, gentamycin, ampicillin, tetracycline, chloramphenicol, streptomycin, ciprofloxacin, and penicillin G. At a level of 1×10^5 CFU each respective strain was inoculated onto MRS agar containing serially diluted antibiotics. Incubation was at 37 °C for 24 h. The minimal inhibitory concentration (MIC) values were compared with the breakpoint values of each strain as suggested by Danielsen and Wind (2003), EFSA (2012), and SCAN (2002), as described in Table 3.

2.6. Phenol resistance

The resistance of each strain to phenol was performed as previously described by Ji et al. (2013). Briefly, bacterial strains grown for 16 h were inoculated in MRS broth with or without 0.4% phenol and incubated at 37 °C. *Lactobacillus rhamnosus* strain GG was used as reference. Using serial dilutions, the numbers of viable cells were counted on MRS agar plates both at time points 0 and 24 h after inoculation.

2.7. Adaptation to cabbage juice

For the *in situ* simulation of the raw material, cabbage juice was prepared by blending Chinese cabbage with distilled water at a ratio of 1:1 (m/v) and filtered two times using both cloth and qualitative paper filter No. 2 (Advantec Toyo, Japan). The prepared cabbage juice was used immediately for the experiment. *Lactobacillus* strains including LGG, grown for 16 h in MRS broth, were centrifuged at 12,000 × g for 3 min and the pellets suspended in cabbage juice. A level of 1×10^5 to 1×10^6 viable cells of each strain was used for inoculation into each cabbage juice sample containing 0% and 2% of NaCl (m/v) respectively. Every sample was incubated at 37 °C, the pH measured and the number of viable cells counted after 24, 48 and 72 h, respectively.

2.8. Response to simulated stomach duodenum-passage

The method of Mathara et al. (2008) was followed. Briefly, strains grown for 16 h in MRS broth at 37 °C were inoculated at a level of 1% into MRS broth at pH 3.0 and incubated at 37 °C. After 1 h of incubation, duodenum juice and bile acid prepared as previously described (Mathara et al., 2008) were added immediately and the media incubated at 37 °C for two more hours. The number of viable bacterial cells was counted after 0 h, 1 h and 3 h.

2.9. Animal experiments

The animal experiments were approved by the Committee on the Ethics of Animal Experiments of Handong Global University. 7-week-old C57BL/6J male mice supplied by Koatec (Gyeonggi, Korea) were housed at 23 ± 1 °C and 55± 10% humidity, in a 12 h light/dark cycle and provided with filtered water and 60 Kcal % fat rodent diet #D12492 (Research Diet, USA) *ad libitum* for 10 weeks. Each group, comprising five mice, was housed in a single cage. After three weeks of adaptation, 1×10^8 CFU viable cells of a strain mixture (50:50 of *L. plantarum* HAC01 and *L. sakei* HAC13) suspended in 20 μL of PBS were given to the mice once a day for 10 more weeks. Sterile PBS without bacteria was administered to the control groups (LF + PBS and HF + PBS). The commercially used probiotic strain, *L. rhamnosus* GG, was used as reference strain and administered in the same way. After growth for 16 h in MRS broth at 37 °C, each LAB strain was prepared daily by centrifugation at 16,000×*g* for 5 min and duplicate washing with sterile PBS. The weight of each animal and its feed consumption were measured once a week. At the end of experimental period, animals were sacrificed by cervical dislocation. Epididymal adipose depots were collected and their weight measured.

2.10. Statistical analysis

Data are expressed as means ± SD. Students' t-test was used for comparing data from different groups. Statistical analyses were performed using the GraphPad Prism Program (version 5.01, GraphPad Software Inc., USA). Significance was accepted at $P < 0.05$.

3. Results

3.1. Isolation of potentially functional strains from white kimchi

LAB strains were isolated from six different samples of white kimchi fermented for 10–15 days. The pH of samples ranged between 3.8 and 4.0 with an average total number of bacterial colonies of 4×10^8 CFU/mL detected on MRS agar plates (Table 1). The

Table 1
Identity of 13 different dominant *Lactobacillus* strains isolated from six samples of Korean white kimchi. Estimation of strain populations was based on matching colony morphology at the highest dilution levels and microscopic confirmation. The viable populations were determined at 37 °C, as selective indication of strains with the potential to grow at body temperature.

Sample	Total number of bacterial colonies (CFU/mL)	pH of sample	Population levels[a] (CFU/mL)	Species and strain no.
A	9.7×10^8	3.8	1.1×10^8	*Lactobacillus plantarum* HAC01
			7×10^7	*L. plantarum* HAC02
B	4.7×10^8	3.94	1×10^7	*L. plantarum* HAC03
C	5.3×10^7	3.98	3×10^6	*L. sakei* HAC04
D	2.3×10^7	3.92	4×10^6	*L. sakei* HAC05
E	3.0×10^8	3.86	1×10^8	*L. brevis* HAC06
F	7.4×10^8	3.9	4×10^8	*L. plantarum* HAC07
			3×10^8	*L. brevis* HAC08
			1.1×10^8	*L. brevis* HAC09
			1×10^7	*L. sakei* HAC10
			2×10^7	*L. sakei* HAC11

[a] At 37 °C.

number of strains in each sample was determined according to the macroscopic (colony morphology) and microscopic (cell morphology) characteristics. Following confirmation of negative catalase reaction and 16S rRNA sequencing analysis, only lactobacilli were selected among the various LAB strains for further investigations. These comprised four different strains of *L. plantarum*, one of *L. sakei* and three of *L. brevis* (Table 1).

3.2. Safety evaluation

To determine the safety of the isolated *Lactobacillus* strains, haemolytic activity and gelatine hydrolysis were examined for the 11 strains none of which showed any haemolysis or gelatine hydrolysis activity (data not shown). *Bacillus cereus* ATCC27348 served as positive control in both tests.

Biogenic amines (BA) are typically associated with fermented foods, and the consumption especially of histamine and tyramine may cause food intoxication and allergic responses with symptoms such as flushing, headaches, nausea, cardiac palpitations, and blood pressure instabilities (EFSA, 2011). BA formation during food fermentation may vary according to the microbial population and chemical and techno-physical conditions. It is associated with the activity of amino acid decarboxylase positive microorganisms, and the exclusion of BA-positive strains from food biotechnical applications is considered an important selection criterion for safe strains (Halász, Baráth, & Holzapfel, 1999). All *L. brevis* strains were found to produce tyramine and/or histamine from tyrosine or histidine and were therefore excluded from further investigations. No other strain showed any BA formation from any of the precursor amino acids tyrosine, histidine, ornithine and lysine (Table 2).

All BA negative strains were examined for antibiotic resistance, using clinically relevant antibiotics. Of particular importance is the presence of a transferable antibiotic resistance gene constituting the risk of transfer to harmful bacteria in the intestine. Two strains of *L. sakei* (HSC 04 and HSC 05) showed low-level ampicillin resistance when compared to the minimal inhibitory concentration suggested by Danielsen and Wind (2003) that was based on a relatively comprehensive study on antibiotic resistance in *Lactobacillus* spp. Thus far, studies on breakpoint values for LAB have not included all food relevant species, resulting in some controversies on official breakpoint values for some species; in addition, intra-species variations on strain level are not uncommon. Thus, for additional reference information, the breakpoints for both *L. plantarum* and *L. sakei* suggested by SCAN (2002) and EFSA (2012) are also shown in Table 3, but, due to the still existing insufficient information on intra-species variations, were not considered as absolute criterion for determining resistance of strains in this study. Strains with BA formation activity and showing antibiotic resistance above the levels suggested by Danielsen and Wind (2003) were excluded from subsequent studies. However, *L. sakei* HAC05, showing low resistance to ampicillin, was included in further investigations as we considered its level of resistance not significant (Table 3).

Table 2
Biogenic amine production of each LAB strain isolated from different samples of white kimchi, detected on a modified MRS medium with the specific precursor amino acids shown.

Strain name	Biogenic amine production from precursor amino acid:			
	Tyrosine	Histidine	Ornithine	Lysine
L. plantarum HAC01	−	−	−	−
L. plantarum HAC02	−	−	−	−
L. plantarum HAC03	−	−	−	−
L. sakei HAC04	−	−	−	−
L. sakei HAC05	−	−	−	−
L. brevis HAC06	+	−	−	−
L. plantarum HAC07	−	−	−	−
L. brevis HAC08	+	++	−	−
L. brevis HAC09	++	+	−	−
L. sakei HAC10	−	−	−	−
L. sakei HAC11	−	−	−	−

++: strong significant colour change, +: significant colour change, −: no significant colour change of bromocresol purple in BA production detection agar.

Table 3
Minimum inhibitory concentration (MIC) in μg/mL of antibiotics for *Lactobacillus* strains isolated from kimchi. Eight different antibiotics were used: Em = erythromycin; Gm = gentamycin; Am = ampicillin; Te = tetracyclin; Ch = chloramphenicol; Sm = streptomycin; Ci = ciprofloxacin. Values above the breakpoint suggested by Danielsen and Wind (2003) are shown in bold.

Species/Strains	Minimum inhibitory concentration (μg/mL)							
	Em	Gm	Am	Te	Ch	Sm	Ci	Pe
L. plantarum								
HAC01	<0.25	32	2	64	4	>128	32	2
HAC02	0.5	32	1	32	4	>128	16	0.5
HAC03	<0.25	32	2	64	4	>128	32	2
HAC07	<0.25	32	<0.25	16	4	8	32	<0.25
Breakpoint for *L. plantarum* according to:								
Danielsen and Wind (2003)	4	128	4	64	16	>256	>32	4
SCAN (2002)	4	1	2	16	16	16	4	2–8
EFSA (2012)	1	16	2	32	4	n.r.[a]	n.i.[b]	n.i.
L. sakei								
HAC04	<0.25	32	**16**	1	2	8	32	<0.25
HAC05	<0.25	32	**8**	2	1	>128	16	<0.25
HAC10	1	32	1	8	2	>128	16	0.25
HAC11	<0.25	32	4	2	1	>128	16	<0.25
Breakpoint for *L. sakei* according to:								
Danielsen and Wind (2003)	1	128	4	8	16	>256	>32	4
SCAN (2002)	4	1	2	16	16	16	4	2–8
EFSA (2012) [c]	1	16	4	8	4	64	n.i.	n.i.

[a] n.r. = not required.
[b] n.i. = no information was provided.
[c] Breakpoints for facultatively heterofermentative *Lactobacillus* spp., as EFSA (2012) did not provide a specific breakpoint for *L. sakei*.

3.3. Adaptation to cabbage juice

As the strains were isolated from kimchi, a fermented vegetable product, we expected their general adaptation to Chinese cabbage, the most typical raw material used. Juice samples extracted from fresh Chinese cabbage (*B. rapa*) and were used for the test with or without 2% of NaCl; kimchi is usually processed with relatively high salt content, ranging between 2 and 3% (w/v) after equilibration. Interestingly, all strains, even including LGG, showed better growth in presence of 2% salt compared to salt-fee cabbage juice. Only two strains, *L. plantarum* HAC01 and *L. sakei* HAC10, showed significant fermentation of salt-free cabbage juice within 24 h with no further growth increase over the next 24 h. *L. plantarum* HAC07 did not grow in the salt-free sample within the first 24 h, but showed increase in population after 48 h from inoculation (data not shown). Likewise, the strongest decrease in pH values were also measured for all strains grown with 2% NaCl (Table 4). Yet, extreme strain-specific differences were found in growth abilities over the incubation period of 24 h, with three *L. plantarum* strains, two *L. sakei* and the LGG strain not showing any growth increase within 24 h in salt-free cabbage juice (Table 4).

3.4. Resistance to 0.04% phenol and ability to hydrolyse bile salt

Phenols comprise one possible antimicrobial factor that

Table 4
Adaptation of *Lactobacillus* strains, isolated from kimchi, to cabbage juice with and without salt. Data shown represent mean ± standard deviation (SD). Each experiment was performed in duplicate. *L. rhamnosus* GG served as reference probiotic strain.

Species/Strain	% of NaCl	pH		Log CFU/mL		
		0 h	24 h	0 h	24 h	Change in log units
L. plantarum						
HAC01	0	6.89	4.17 ± 0.04	5.80 ± 0.00	7.78 ± 0.04	1.98
	2	6.62	3.66 ± 0.01	5.78 ± 0.00	8.29 ± 0.08	2.5
HAC02	0	6.89	6.45 ± 0.00	5.51 ± 0.15	<5.00	n.g.[a]
	2	6.61	4.17 ± 0.01	5.56 ± 0.06	8.33 ± 0.01	2.78
HAC03	0	6.87	6.48 ± 0.02	5.85 ± 0.01	<5.00	n.g.
	2	6.58	4.29 ± 0.06	5.93 ± 0.14	8.29 ± 0.08	2.36
HAC07	0	6.86	6.22 ± 0.04	5.80 ± 0.20	<5.00	n.g.
	2	6.6	3.53 ± 0.09	5.73 ± 0.13	8.25 ± 0.15	2.52
L. sakei						
HAC05	0	6.84	6.42 ± 0.05	5.69 ± 0.10	<5.00	n.g.
	2	6.58	4.25 ± 0.01	5.63 ± 0.04	8.09 ± 0.12	2.46
HAC10	0	6.78	4.07 ± 0.04	5.38 ± 0.58	7.26 ± 0.08	1.88
	2	6.55	4.09 ± 0.01	4.96 ± 0.02	7.78 ± 0.05	2.82
HAC11	0	6.75	5.91 ± 0.03	5.08 ± 0.56	<5.00	n.g.
	2	6.56	3.53 ± 0.06	4.65 ± 0.02	8.27 ± 0.05	3.61
L. rhamnosus						
GG	0	6.78	6.20 ± 0.05	5.79 ± 0.04	<5.00	n.g.
	2	6.53	3.59 ± 0.02	5.81 ± 0.04	8.10 ± 0.06	2.7

[a] n.g. = no growth.

Lactobacillus strains in the intestinal environment need to overcome; these phenols may be generated from activities of gut microbiota (Xanthopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2000). To assess growth and/or survival in the presence of phenol, strains were grown in MRS broth with or without 0.4% phenol at 37 °C. All strains were relatively sensitive to phenol compared to the LGG strain (Table 5). No inter-species differences in phenol resistance were noticeable between the two species, *L. plantarum* and *L. sakei*, even when *L. plantarum* strains (except for *L. plantarum* HAC02) grew better without phenol. Interestingly, the stains showing the lowest survival in presence of phenol, *L. plantaum* HAC07, *L. sakei* HAC10, and *L. sakei* HAC11, were all isolated from sample F (Table 1).

Bile salt hydrolysis of LAB strains is considered to one of the key functional properties associated with a reduced absorption of bile salt in the intestine. Thereby the re-conjugation of bile salt is blocked and the utilisation of cholesterol as precursor for the *de novo* bile salt synthesis is increased. All strains generated a precipitation zone in the medium with taurocholic acid, indicating their ability to hydrolyse bile salts, and suggesting a possible cholesterol lowering effect by de-conjugation of bile salt (Dashkevicz & Feighner, 1989).

3.5. Response to conditions simulating the stomach duodenum-passage

The possible survival rate of the potentially probiotic strains was evaluated on the basis of their viability under *in vitro* passage of simulated stomach and duodenum conditions (SSDP). In our study, *L. plantarum* survived at a much higher rate than *L. sakei* strains and even the probiotic LGG strain. Most of the *L. plantarum* strains showed high resistance to low pH (3.0), but exposure to high concentrations of oxgall decreased the numbers of surviving cells. The viable cell count numbers of *L. sakei* also decreased more under the oxgall conditions than in the acidic environment. Among the isolated *Lactobacillus* strains, *L. plantarum* HAC01 showed the highest survival rate being even 35 times higher than that of LGG, used as a reference probiotic strain (Table 6).

3.6. Animal experiments

To assess the effect of isolated strains on abnormal host status, the strains *L. plantarum* HAC01 and *L. sakei* HAC11, showing the highest survival among the strains of each of these species in SSDP, were selected for use in the animal experiments and for

Table 5
Resistance of *Lactobacillus* strains isolated from kimchi to 0.4% (v/v) phenol. The data are shown as mean ± standard deviation (SD). Each experiment was performed in duplicate. *L. rhamnosus* GG served as reference probiotic strain.

Species/Strains	Viable counts (Log CFU/mL)					
	MRS broth			MRS broth + 0.04% phenol		
	0 h	24 h	Change in log units	0 h	24 h	Change in log units
L. plantarum						
HAC01	7.33 ± 0.01	9.41 ± 0.04	2.07	7.21 ± 0.09	6.22 ± 0.02	−1.00
HAC02	7.37 ± 0.07	9.09 ± 0.12	1.73	7.28 ± 0.11	6.42 ± 0.14	−0.88
HAC03	7.34 ± 0.15	9.56 ± 0.03	2.22	7.33 ± 0.10	7.25 ± 0.24	−0.08
HAC07	6.37 ± 0.01	8.67 ± 0.02	2.30	6.41 ± 0.05	4.40 ± 0.13	−2.14
L. sakei						
HAC05	6.33 ± 0.01	7.77 ± 0.02	1.43	6.13 ± 0.02	5.45 ± 0.05	−0.68
HAC10	6.41 ± 0.04	7.84 ± 0.04	1.43	6.58 ± 0.05	5.18 ± 0.00	−1.40
HAC11	6.34 ± 0.06	8.26 ± 0.12	1.92	6.22 ± 0.01	4.17 ± 0.12	−2.05
L. rhamnosus						
GG	8.84 ± 0.09	8.84 ± 0.00	2.00	6.82 ± 0.12	7.77 ± 0.06	0.95

Table 6
Response of strains isolated from kimchi to *in vitro* conditions simulating stomach and duodenum passage. The data are shown as mean ± standard deviation (SD). Each experiment was performed in duplicate. *L. rhamnosus* GG served as reference probiotic strain.

Species/Strain	Initial count (log CFU/mL)	After one hour in pH 3.0 (log CFU/mL)	After two more hours in 1.3% oxgal (log CFU/mL)	Overall survival rate (%)
L. plantarum				
HAC 01	9.50 ± 0.029	9.24 ± 0.195	8.78 ± 0.087	19.38 ± 5.121
HAC 02	9.58 ± 0.032	9.21 ± 0.154	8.74 ± 0.056	14.44 ± 0.786
HAC 03	9.49 ± 0.020	9.42 ± 0.035	8.76 ± 0.011	18.72 ± 0.398
HAC 07	9.11 ± 0.047	8.81 ± 0.061	7.55 ± 0.089	2.76 ± 0.135
L. sakei				
HAC 05	8.86 ± 0.042	7.30 ± 0.062	5.82 ± 0.083	0.09 ± 0.026
HAC 10	8.69 ± 0.006	7.37 ± 0.066	4.70 ± 0.055	0.01 ± 0.001
HAC 11	8.87 ± 0.012	7.61 ± 0.045	5.88 ± 0.000	0.10 ± 0.003
L. rhamnosus				
GG	9.12 ± 0.311	8.54 ± 0.281	6.82 ± 0.064	0.54 ± 0.293

comparison with the LGG strain, serving as reference probiotic strain. Strains HAC01 and HAC11 were administered as a 50:50 mixture to C57BL/6J male mice fed on a high fat (HF) diet (60% kcal fat, Research Diet, USA) for 8 weeks. Each animal group receiving the strain mixture (HF + MX) and LGG (HF + LG) showed significantly lower body weight and total weight gain during 8 weeks compared to the high-fat control group (HF + PBS) (Fig. 1 A and B). Moreover, both the strain mixture and LGG noticeably reduced epididymal and mesenteric adipose depots even when these differences were not statistically significant (Fig. 1 C and D). There was no significant difference in liver weight among all groups including a group given low-fat diet (10% kcal, Research Diet, USA) instead of HF diet (LF + PBS) (Fig. 1C).

4. Discussion

In this study, eleven different *Lactobacillus* strains were isolated from Korean white kimchi and evaluated for their safety and functionality in view of their potential use as probiotics. The pH value of the six kimchi samples ranged from 3.8 to 3.98, and the total number of viable microorganisms in these samples varied between 2.3×10^7 and 9.7×10^8 cfu/mL, with LAB dominating the population, indicating that the fermentation process had progressed sufficiently (Kang, Lee, Min, & Min, 2003; Rhee, Lee, & Lee, 2011). According to previous studies, *Leuconostoc* species typically predominate the first stage of kimchi fermentation, and are frequently accompanied or succeeded by *Weissella*. With decreasing pH *Lactobacillus* species such as *L. plantarum, L. brevis* and *L. sakei* soon constitute the dominating population (Cho et al., 2006; Kim, Lee, Park, Kim, & Han, 2000; Rhee et al., 2011); this domination has also been confirmed by our present study. In case of white kimchi, So and Kim (1997) reported that the pH changed from 6.15 to 3.40 over a fermentation period of 50 days. The initially dominating microbial population of Gram-negative rods - comprising *Pseudomonas*, *Enterobacter* and *Erwinia* – was succeeded by *Leuc. mesenteroides*, *Leuc. paramesenteroides*, and followed by *L. plantarum* and *L. brevis*.

The three *L. brevis* strains were isolated from two different samples of kimchi at levels of $> 10^8$ cfu/mL, and have shown ability to produce biogenic amines either from tyrosine only, or both

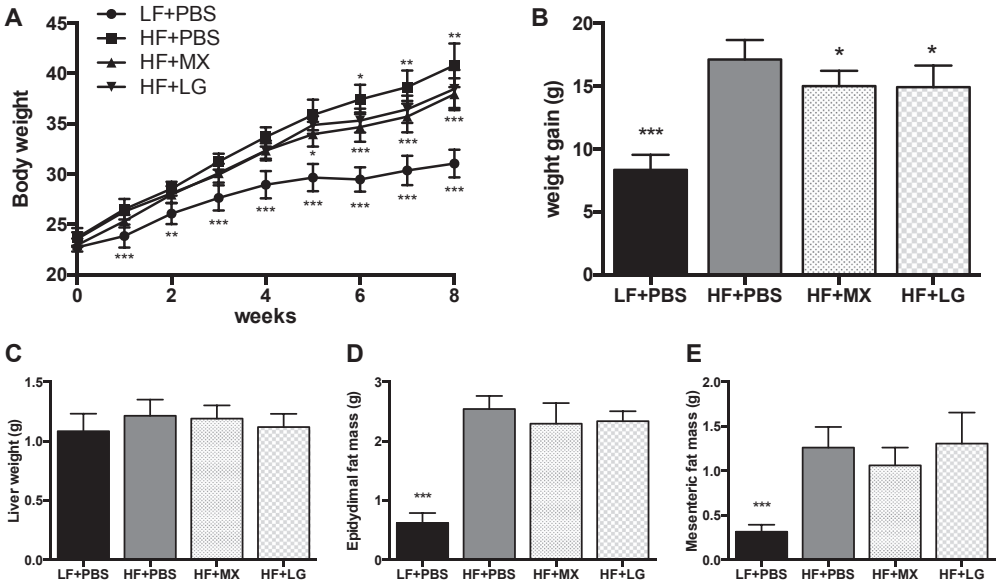

Fig. 1. Results of an *in-vivo* experiment with administration of selected *Lactobacillus* strains to 7-week-old C57BL/6J male mice receiving a high-fat (HF) diet over 10 weeks following a 3-weeks adaptation period. The groups comprised 5 animals per variable. The body weight (A) and total weight gain (B) during an eight weeks feeding period are shown; liver weight (C), epididymal fat mass (D), and mesenteric fat mass (F) were measured at the end of the experiment. (N = 4–5). LF = low-fat diet; HF = high-fat diet. A mixed strain culture *of L. plantarum* HAC01 and *L. sakei* (MX) or the single *L. rhamnosus* LGG strain (LG) were suspended in PBS; sterile PBS was administered to both control groups (LF + PBS and HF + PBS). The data are shown as mean ± SD and were analysed with two-way or one-way ANOVA compared with the HF + PBS group. *P < 0.05, **P < 0.01, **P < 0.001 by Fisher's LSD test.

tyrosine and histidine, while for strains of *L. plantarum* and *L. sakei* no amino acid decarboxylase activity could be detected with the precursor amino acids provided. Biogenic amines naturally occur only in low amounts in several natural foods without fermentation. They are frequently produced during food fermentations, mainly by microbial amino acid decarboxylase activities under varying conditions (Halász, Barath, Simon-Sarkadi, & Holzapfel, 1994; 1999), thereby also posing a potential health hazard in contrast to the otherwise beneficial properties of fermented foods (Önal, 2007). *L. brevis* has been reported as one of the strongest tyramine producers among the *Lactobacillus* species while, on the other hand, most *Lactobacillus* strains do not produce any cadaverine or putrescine (Bover-Cid & Holzapfel, 1999). Even though most commercial kimchi products contained safe levels of biogenic amines (Mah, No, Kim, & Hwang, 2004), Tsai et al. (2005) reported high histamine concentrations in kimchi products in Taiwan reaching average levels of 49.8 mg/100 g, greatly exceeding the proposed safety level of 5 mg/100 g by US Food and Drug Administration. Strains of *L. brevis*, *L. paracasei* and *Brevibacillus brevis* were identified as major bacteria capable of producing histamine (Tsai et al., 2005).

For antibiotic resistance examination, only the *L. sakei* strains HAC04 and HAC05 have shown a possibly acquired resistance to amoxicillin based on the cut-off value suggested by both Danielsen and Wind (2003) and EFSA (2012). Previous studies have reported considerable differences between cut-off values for the inhibition of *L. plantarum* by gentamycin. With reference to the EFSA (2012) suggestions, our *L. plantarum* strains HAC01 and HAC03 showed only mild resistance and were thus considered as susceptible according to the breakpoint values suggested by Danielsen and Wind (2003). Antibiotic resistance has been reported for food-associated LAB; most of the relevant food products were associated with either milk fermentations or with raw meat products in which prevalent supplementation of antibiotics as growth promoter for animals has been identified as a major factor (Mathur & Singh, 2005).

In this study, clear differences were found between strains of *L. plantarum* and *L. sakei*, the major species associated with fermented kimchi, with regard to both acidic and bile stress conditions in the *in vitro* SSDP experiment. *L. plantarum,* with a genome size of >3 million bp (Siezen et al., 2012), showed higher resistance than *L. sakei* (genome size $\leq$ 2 million bp) (Chaillou, Lucquin, Najjari, Zagorec, & Champomier-Vergès, 2013) under both these stress conditions. The average survival rate of *L. plantarum* strains was more than 200 times higher than that of *L. sakei* strains and still around 30 times higher than that of LGG (genome size 3,010,111 bp; Morita et al., 2009). Still, strains of *L. plantarum* and *L. sakei* did not show any marked differences in salted cabbage juice fermentation and neither in their resistance to phenol. It is interesting that some strains of either species, including LGG, were not able to ferment cabbage juice without salt; e.g., *L. plantarum* strains HAC01 and HAC02 were isolated from the same kimchi sample, but strongly differed in their adaption to non-salty cabbage juice with only HAC01 showing growth increase of two log units within for 24 h, and with 1.5 logs increase only for *L. sakei* strain HAC10. On the other hand, 2% salt appeared to be essential for all tested strains, for which optimal growth increases of 2.5–3 log units were detected within 24 h. These results imply that only some strains such as *L. plantarum* (HAC01) and *L. sakei* (HAC10) may be adapted to low osmotic pressures, while stress-related conditions such as elevated salt concentrations appear to favour the tested LAB strains, including LGG.

A preliminary study was conducted on the anti-obesity effect of a mixture of the two selected strains (*L. plantarum* HAC01 and *L. sakei* HAC10) using a diet-induced obesity murine model. With this *in-vivo* study, it was attempted to obtain insight into the effect of consumption of kimchi in relation to representative strains typical of its LAB population, rather than focusing on the specific functionality of each individual strain. Even when a significant decrease of fat mass could not be detected, due to the small number of experimental animals in each group (N = 4–5), the weight gains during the experimental period of 8 weeks were significantly lower than for the positive control. Moreover, both the epididymal and mesenteric fat mass was also noticeably reduced compared to the control group. These results suggest a positive influence of kimchi consumption on obesity, an increasing problem in industrialised countries due an unbalanced diet pattern and modern lifestyle. This effect seems to be strongly related to the LAB strains associated with the fermented kimchi.

The low survival rate of the *Lactobacillus* strains in 0.4% phenol is considerable in terms of their actual activity in the host gastrointestinal tract (GIT). Even when the relevance of the *in vivo* activities to phenolic compounds in the GIT may be uncertain, the administration of the mixture of the two isolated strains has shown impact on animals similar to that of LGG, which was more resistant to phenol (Table 5). Yet, the strain mixture showed a noticeable decrease in mesenteric adipose depot as compared to LGG and the positive control. This still leaves open the important question on the mechanism of beneficial LAB interaction in our GIT. LAB may promote beneficial (direct and indirect) effects by their metabolic activities in the gut. In addition, these effects may be also caused by ecological changes in the GIT environment and modulation of gut microbiota, even by their passage through the intestinal tract instead of colonising and direct interaction with the epithelium (Brigidi, Vitali, Swennen, Bazzocchi, & Matteuzzi, 2001; Kajander et al., 2008; Ohland & MacNaughton, 2010; Pessione, 2012). These studies have opened a wider perspective for future investigations into the mechanisms of beneficial LAB activities in the body, thus including not only direct but also indirect and multidirectional influences to the host. This poses a great challenge towards a deeper understanding of microorganism:host interactions. Approaches should include consideration of specific properties by which LAB strains involved in food fermentations such as kimchi may be distinguished from other strains of the same species.

Acknowledgements

This research was supported by Korea Institute of Planning and Evaluation Technology (project no. 911053-1) in Food, Agriculture, Forestry and Fisheries (IPET).

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