

ORIGINAL ARTICLE

***Lactobacillus kunkeei* YB38 from honeybee products enhances IgA production in healthy adults**T. Asama¹, T.-H. Arima², T. Gomi², T. Keishi¹, H. Tani¹, Y. Kimura¹, T. Tatefuji¹ and K. Hashimoto¹¹ Institute for Bee Products and Health Science, Yamada Bee Company, Inc., Kagamino-cho, Tomata-gun, Okayama, Japan² Department of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima, Japan**Keywords**bee pollen, honeybee products, Immunoglobulin A, *Lactobacillus kunkeei*, microbiota.**Correspondence**Takashi Asama, Institute for Bee Products and Health Science, Yamada Bee Company, Inc. 194 Ichiba, Kagamino-cho, Tomata-gun 708-0393, Japan.
E-mail: ta1022@yamada-bee.com

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Abstract**Aims:** To identify lactic acid bacterial isolates, which promote immunoglobulin A (IgA) production in honeybee products and honeybees (*Apis mellifera*).**Methods and Results:** Pyrosequencing analysis of the microbiota of honeybee products and honeybees revealed the predominance of *Lactobacillus kunkeei* in honey, bee pollen, bee bread and royal jelly. *Lactobacillus kunkeei* was isolated from bee pollen, bee bread and honey stomach, and its effect on IgA production was evaluated *in vitro*. Heat-killed YB38 and YB83 isolates from bee pollen promoted IgA production in mouse Peyer's Patch cells and had little mitogenic activity or effect on IL-2 production in mouse spleen cells in comparison with *Listeria monocytogenes*, which does exhibit mitogen activity. A pilot study in 11 healthy adults showed that 4-week intake of 1000 mg day⁻¹ heat-killed YB38 increased secretory IgA (SIgA) concentrations and secretion in saliva with no adverse effects.**Conclusion:** Heat-killed *Lact. kunkeei* YB38 from bee pollen increases IgA production and may safely improve immune responsiveness.**Significance and Impact of the Study:** This is the first report of microbiota analysis of royal jelly and the immune efficacy of *Lact. kunkeei* from honeybee products in humans.**Introduction**

Honeybee (*Apis mellifera*) products such as honey, royal jelly, bee pollen and bee bread have long been used as folk medicine. Bee bread, a fermented food for worker bees and their larvae, is a mixture of bee pollen and honey stored in beehives for several weeks; lactic acid bacteria (LAB) are considered important for this fermentation (Vasquez and Olofsson 2009). LAB species in honeybee products have been identified by microbiota analysis, which showed *Lactobacillus kunkeei* to be the dominant species in honey, bee pollen and bee bread (Anderson *et al.* 2013; Corby-Harris *et al.* 2014). Various kinds of LAB have been isolated from honeybee products, including *Lact. kunkeei*, *Lactobacillus* sp., and *Bifidobacterium* sp. from bee pollen and bee bread (Vasquez and Olofsson 2009; Anderson *et al.* 2013). *Enterococcus faecium* has also been isolated from honey (Olofsson and Vásquez 2008; Iburguren *et al.* 2010). Bacteria isolated from honeybee products exhibit antimicrobial activity; however, other potential human benefits have not been explored.

Some strains of LAB are considered beneficial for humans, with activities such as normalization of gut microbiota (Fukushima *et al.* 1997) and anti-allergic effects (Ishida *et al.* 2005). They also influence the mucosal immune system, such as in the intestine, promoting immunoglobulin A (IgA) production in Peyer's patch cells (Kobayashi *et al.* 2011). LAB-mediated immunomodulation tends to be stronger with heat-killed than live bacteria (Izumo *et al.* 2011).

We explored the microbiota of honeybee products and the immunomodulatory effects of heat-killed *Lact. kunkeei* in humans.

Materials and methods**Microbiota analysis of honeybee products and honeybees***Sample collection and pretreatment*

Honeybee products and honeybees were collected from the apiary at the Yamada Bee Company in Okayama, Japan, between September and October. Whole guts and honey

stomachs were dissected from five randomly selected bee workers. These samples were placed in sterile 1.5-ml tubes containing phosphate-buffered saline (PBS, pH 7.4) and homogenized with a pestle (As One, Osaka, Japan). The honey stomach homogenates were washed with PBS, centrifuged at 9000 *g* for 10 min at 4°C, then resuspended in 3 ml TE10 buffer (10 mmol l⁻¹ Tris, 10 mmol l⁻¹ EDTA, pH 8) for DNA extraction. Whole guts containing bee pollen were filtered to remove bee pollen, because DNA extracts from plants produce amplicons with the universal primers for 16S rRNA genes (Suda *et al.* 2008). The whole-gut homogenates were filtered through a 10- μ m Cell Trics Filter (PARTEC, Münster, Germany), then with a 100- μ m Falcon Yellow Nylon Mesh Cell Strainer (Becton Dickinson, Franklin Lakes, NJ) to remove gut tissue and pollen. Then, bacteria from whole guts were resuspended in 3 ml TE10 buffer for DNA extraction. Samples of bee pollen, bee bread and royal jelly (1 g) were suspended in 2 ml PBS and treated in the same manner as the whole guts. A 1 g sample of honey was suspended in 5 ml PBS and centrifuged at 9000 *g* for 10 min at 4°C, then resuspended in 3 ml TE10 buffer for DNA extraction.

DNA extraction and RNase treatment

DNA extraction was performed as described (Kim *et al.* 2013). The DNA pellets were dissolved in 25 μ l TE buffer (10 mmol l⁻¹ Tris, 1 mmol l⁻¹ EDTA, pH 8) and incubated at 37°C for 30 min with 2.8 μ l RNase (Wako Pure Chemical Industries, Osaka, Japan). The solutions were mixed with an equal volume of 20% PEG (PEG 6000-2.5 mol l⁻¹ NaCl) on ice for 10 min. DNA was pelleted by centrifugation at 25 000 *g* at 4°C for 10 min, then rinsed with 75% ethanol, dried *in vacuo* and dissolved in TE buffer.

PCR and pyrosequencing

Analysis of 16S sequences and operational taxonomic units was performed as described, with the exception of the chimera check (Kim *et al.* 2013). All sequences were deposited in DDBJ/GenBank/EMBL under accession number DRA003110.

Isolation of LAB

Each sample (honey stomach, bee pollen, bee bread and royal jelly) was placed in sterile distilled water and suspended with a homogenizer. Homogenates were anaerobically incubated in MRS liquid medium (Oxoid, Hampshire, UK) at 25–30°C for 1–2 days. After incubation, each culture was inoculated into MRS agar, and anaerobically incubated at 25–30°C for 5–7 days. Bacterial colonies were picked at random (10–30) and subcultured to obtain pure isolates. LAB were incubated at 30–37°C for 2–7 days on MRS agar with 1.0% (weight per

volume) CaCO₃ (Kopermsub and Yunchalard 2010). Acid-producing bacterial colonies were isolated from the agar plates. All isolates were subcultured to obtain pure isolates that were stored at –80°C in a freezing medium.

Bacterial identification by 16S rRNA gene sequencing

Strains were cultivated on MRS agar at 30°C for 2 days. DNA was extracted by suspending a single colony in 100 μ l TE buffer and heating at 99°C for 10 min. The crude extracts were centrifuged at 20 000 *g* for 5 min and the supernatant was used directly as a template for PCR. Amplification was performed using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') for full length identification and 341F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') for partial identification. PCR was performed on a TProfessional Basic Gradient (Biometra, Gottingen, Germany) under the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 90 s, with a final extension at 72°C for 5 min. The amplified product was purified using an UltraClean PCR Clean-up DNA purification kit (MO BIO Laboratories, Carlsbad, CA). Cycle-sequencing reactions were performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Japan, Tokyo, Japan) with 341F and 907R primer for partial identification. For full length identification, we used primers 27F, 520R (5'-ACCGCGGCTGCTGGC-3'), 520F (5'-GTGCCAGCAGCCGCGG-3'), 1100R (5'-AGGGTTGCGCTCGTTG-3'), 1100F (5'-GCAACGAGCGCAACCC-3') and 1492R (5'-GGTTACCTTGTACGACTT-3'). An Applied Biosystems 3130xl Genetic Analyzer (Life Technologies Japan) was used for sequence determination. Homology searches of the 16S rRNA sequences were performed in GenBank with the BLAST program.

In vitro screening for immunomodulation

IgA production in Peyer's patch cells

Animal experiments were performed according to the animal care guidelines of Kitasato University. Thirty five-week-old female BALB/c mice (Japan SLC) were euthanized by intraperitoneal administration of pentobarbital sodium (50 mg kg⁻¹, Kyoritsu Seiyaku Corporation, Tokyo, Japan). Individual Peyer's patches (PP) were cut from the intestine of BALB/c mice with curved scissors and cells were suspended in Hank's balanced salt solution (Life Technologies, Carlsbad, CA). Dissociated PP cells were washed three times by 110 *g* centrifugation for 5 min at 4°C, then resuspended in RPMI 1640 (Life Technologies) supplemented with 10% foetal bovine serum (FBS; Life Technologies) at 1 \times 10⁶ cells ml⁻¹. PP cells were cultured in 24-well Cell

Culture Clusters (Costar3526, Corning) containing 2 ml RPMI 1640, 10% FBS and 10 μ l *Lact. kunkeei* suspension (approximately 1×10^5 cells ml⁻¹) at 37°C and 5% CO₂. *Lactobacillus kunkeei* were cultured in Mueller–Hinton agar and heat-killed at 100°C for 30 min. Anti-IgA positive mouse serum (Housekeeping serum) was used as a positive control (final conc. 250 ng ml⁻¹); *Listeria monocytogenes* (Gifted by Kikuo Nomoto, Medical Institute of Bioregulation, Kyushu University) was used as the control strain (1×10^5 cells ml⁻¹). Two days after culture initiation, the culture supernatants (SC) were tested for IgA antibody.

Each well of the ELISA plate (96-well flat-bottom plates, Corning) was coated with 50 μ l goat affinity-purified antibody to mouse Ig (MP Biomedicals, Solon, OH) at 4°C for 16 h. After blocking with 50 μ l per well of 1% BSA-PBS containing 0.05% Tween-20 (PBS-T) (Sigma-Aldrich, St. Louis, MO), SC (50 μ l per well) was added and incubated at 37°C for 1 h. The plates were washed three times with 300 μ l per well PBS-T, and 50 μ l per well peroxidase-conjugated goat affinity-purified antibody to mouse IgA (1 : 2000; alpha chain, MP Biomedicals) was added. After incubation for 1 h at room temperature, the plates were washed, 50 μ l per well FAST OPD was added (Sigma-Aldrich, P9187) and incubated at room temperature for 15 min with light shielding. The reaction was stopped with 50 μ l per well 4 N H₂SO₄ (Wako Pure Chemical Industries, Ltd.) and absorbance was measured at 490 nm with a microplate reader (Benchmark, Bio-Rad Japan, Tokyo, Japan).

Spleen cell proliferation assay

BALB/c mice were euthanized by intraperitoneal administration of pentobarbital sodium. Single-cell suspensions were prepared in Hank's balanced salt solution, washed three times by centrifugation and resuspended in 10 ml RPMI 1640, 10% FBS. The suspension was treated with 33 Gy X-ray irradiation by Linac (LM-6M; Mitsubishi Electric Corp., Tokyo, Japan) and suspended at 1×10^5 cells ml⁻¹ for use as stimulator cells. Un-irradiated single-cell suspensions of 1×10^5 cells ml⁻¹ in RPMI 1640, 10% FBS served as responder cells.

Stimulator and responder cells were mixed in equal volumes in 96-well flat-bottom plates (Costar3596, Corning, Corning, NY). Mixed lymphocyte reactions (MLRs) were performed for 48 h after adding 2 μ l *Lact. kunkeei* suspension (1×10^5 cells ml⁻¹) and 5 μ l 5-bromo-2'-deoxyuridine (BrdU; 5 μ g ml⁻¹, BD Pharmingen, San Diego, CA). Pokeweed mitogen (Sigma-Aldrich, L9379) was used as a positive control (final conc. 5 μ g ml⁻¹), and *L. monocytogenes* was used as the control strain.

Stimulator and responder cells were collected after the MLR and permeabilized with BD Cytofix/Cytoperm and BD Cytoperm Plus Buffer (both from BD Pharmingen),

treated with DNase and stained with Cy5-conjugated anti-BrdU antibodies (BD). Stained cells were analysed with a Cytomics FC500 (Beckman Coulter, Fullerton, CA) and FLOWJO software (Tree Star, Ashland, OR).

CTLL-2 assay

As described in the prior section on spleen cells, MLRs were conducted for 48 h and the supernatant was recovered and used for the CTLL-2 assay.

CTLL-2 cells (5×10^5 cells ml⁻¹; RCB0637, Riken Cell Bank, Tsukuba, Japan) were suspended in RPMI 1640, 10% FBS. A 100 μ l aliquot of cells was seeded in 96-well flat-bottom plates (Costar3596) at 5×10^4 cells per well and 100 μ l of the recovered supernatant was added. Recombinant mouse IL-2 (rmIL-2; Takeda Pharmaceuticals, Osaka, Japan) was used as a positive control (1000 JRU ml⁻¹). BrdU (5 μ l) was added and after 48 h, the cells were collected, fixed and permeabilized, then stained with Cy5-conjugated anti-BrdU antibodies as described above before cell sorting on a Cytomics FC500 with FLOWJO software.

Carbohydrate fermentation test

Carbohydrate fermentation reactions were recorded on an API50CH (bioMérieux, Marcy, France) according to manufacturer instructions. Readings were taken for 2 days at 30°C.

RAPD-PCR amplification

Genomic DNA was extracted from overnight cultures with an UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories) according to manufacturer protocols. Oligonucleotide primer 1254 (5'-CCGCAGCCAA-3') was selected for the reproducibility, distribution, number and intensity of the bands it produced (Torriani *et al.* 1999). Amplification was performed on an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) as follows: preincubation at 94°C for 2 min, followed by 40 cycles of 94°C for 1 min, 30°C for 1 min and 72°C for 1.5 min, and a final extension at 74°C for 5 min (Manan *et al.* 2009). Amplified products were separated by 1% agarose gel electrophoresis in 1× TBE buffer. The gel was visualized and imaged on an LAS 3000 mini (FUJIFILM, Tokyo, Japan) after staining with 10 μ g ml⁻¹ ethidium bromide.

A pilot clinical study to evaluate IgA induction and safety

Heat-killed YB38 preparation

The deposit number of *Lact. kunkeei* YB38 isolated from bee pollen is FERM BP-11439. This strain was cultured at

30°C for 24 h in medium containing yeast extract and high fructose corn syrup. The cells were collected by centrifugation, filtered, heat-killed at 80°C for 1 min and freeze-dried.

Design of human volunteer trials

The study design was approved by the local Ethics Committee (Approval No. 45) and performed in accordance with the Declaration of Helsinki. All subjects provided written informed consent. Subjects with potential food allergies, alcoholism, or severe anaemia were excluded. Twenty-two subjects fit the inclusion criteria and 11 subjects (2 males, 9 females; aged 45.4 ± 8.8 years) were screened in ascending order of SIgA secretion rate ($\mu\text{g min}^{-1}$). This open-label intervention study explored the safety and influence of heat-killed YB38 on SIgA in healthy adults. The subjects took 1000 mg heat-killed YB38 once a day for 4 weeks. Blood and saliva samples were obtained at least 1 h after eating between 14 : 00 and 16 : 00, before and after intake of the heat-killed YB38.

Blood analysis

Haematological indices (leucocytes, erythrocytes, haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, blood platelets, basophils, eosinophils, lymphocytes, monocytes and neutrophils), metabolic markers (plasma glucose, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein cholesterol and triglycerides) and markers of liver and kidney function (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase (LDH), alkaline phosphatase, total protein, albumin, total bilirubin, creatinine, creatinine kinase, urea nitrogen and uric acid) were investigated.

Saliva analysis

Saliva was collected by the passive drool method (Abdullah *et al.* 2009). Each subject rinsed his or her oral cavity a few times with mineral water. Immediately before collection, saliva remaining in the oral cavity was swallowed and the head was immediately inclined slightly forward, and a straw was placed in the mouth to begin saliva collection. Immediately after the start of saliva collection, the subject closed his or her mouth, and saliva was collected into a 15-ml tube for 5 min. Samples were stored at -80°C . SIgA concentration was measured with an ELISA kit (Immundiagnostik, Bensheim, Germany). Total protein concentration was measured with DC Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA). The mean of two measurement values represents the amount of saliva flow.

Statistical analysis

Values are presented as the mean \pm SD. IgA production in PP cells and IL-2 production and mitogen activity in spleen cells were analysed by the Tukey–Kramer test. Blood and salivary parameters were analysed by Student's paired *t*-test.

Results

Community composition of the whole gut, honey stomach and honeybee products based on deep sequencing of 16S rRNA genes

We obtained 12 533 high-quality aligned pyrosequences that were subsequently classified to bacterial genera. The sequences were split between honey (2980), bee pollen (1923), bee bread (756), royal jelly (2275), whole gut (2300) and honey stomach (2299).

Lactobacillus was the dominant component of honey (90.9%), bee pollen (74.6%), bee bread (83.9%), royal jelly (93.3%) and whole gut (34.6%). *Gluconobacter* was the most abundant (64.7%) single genus of the honey stomach (Table 1). *Lactobacillus insectis* was the predominant species in whole guts, while *Lact. kunkeei* was the majority (>98%) species in all honeybee products and made up more than half of the genus *Lactobacillus* in honey stomach (Table 2). Only low numbers of *Lact. kunkeei* were detected in the whole gut.

Isolation and identification of LAB

Following microbiota analysis, we isolated over 1000 bacteria strains from bee pollen, bee bread, royal jelly and honey stomach. After isolation and selection for calcium carbonate solubility, 78 LAB strains were obtained from honey stomach, 50 from bee pollen and nine from bee bread. We were unable to isolate LABs from royal jelly. BLAST analysis of 500-bp 16S rRNA sequences indicated the strains were 98 isolates of *Lact. kunkeei* (bee pollen: 20 strains, bee bread: 9 strains, honey stomach: 78 strains); 30 isolates of *Enterococcus* sp. were obtained only from bee pollen.

Heat-killed *Lactobacillus kunkeei* and IgA production

We performed an *in vitro* screen of 20 *Lact. kunkeei* isolates for their effects on IgA production. BP104 and BP402 produced greater IgA secretion than other *Lact. kunkeei* strains in mouse PP cells (Fig. 1). BB025 showed very weak mitogen activity in mouse spleen cells (Fig. 2a). BB025 and BP402 showed a very weak proliferation effect on IL-2 production by CTLL-2 cells (Fig. 2b). The Z-factor, which is used to evaluate and compare assay quality (Zhang *et al.*

Table 1 Sequence abundance in each sample type

Genus*	Phylum	Average %					
		Honey stomach	Whole gut	Honey	Bee pollen	Bee bread	Royal jelly
<i>Lactobacillus</i>	Firmicutes	22.0	34.6	90.9	74.6	83.9	93.3
<i>Acetobacter</i>	Proteobacteria	0.0	0.0	0.1	0.0	1.2	0.0
<i>Bifidobacterium</i>	Actinobacteria	4.0	21.0	0.0	0.1	0.0	0.1
<i>Burkholderia</i>	Proteobacteria	0.0	0.0	0.0	0.7	2.1	0.0
<i>Chryseobacterium</i>	Bacteroidetes	0.0	0.0	0.0	1.2	0.0	0.0
<i>Curtobacterium</i>	Actinobacteria	0.0	0.0	0.0	1.6	0.0	0.0
<i>Gluconobacter</i>	Proteobacteria	64.7	0.7	7.2	3.4	0.0	2.1
<i>Melissococcus</i>	Firmicutes	0.0	0.0	0.0	0.0	0.3	3.2
<i>Methylobacterium</i>	Proteobacteria	0.0	0.0	0.0	1.6	0.0	0.0
<i>Microbacterium</i>	Actinobacteria	0.0	0.0	0.0	1.2	0.0	0.0
<i>Neisseria</i>	Proteobacteria	2.4	18.2	0.1	0.2	0.0	0.0
<i>Paenibacillus</i>	Firmicutes	0.0	0.0	0.0	2.1	0.0	0.0
<i>Pseudomonas</i>	Proteobacteria	0.0	0.0	0.1	1.1	0.0	0.0
<i>Sphingomonas</i>	Proteobacteria	0.1	0.0	0.0	1.2	0.9	0.0
<i>Stenotrophomonas</i>	Proteobacteria	0.0	0.0	0.0	1.4	0.0	0.0
Others†		6.7	25.5	1.6	9.4	11.6	1.4

*Clustering of 16S rRNA reads was done using a 96% pairwise-identity cut-off with UCLUST (www.drive5.com). Domains of eukaryota were excluded.

†Length coverage is <90%.

Table 2 Abundances of 18 *Lactobacillus* species in each sample type

Species	Average %					
	Honey stomach	Whole gut	Honey	Bee pollen	Bee bread	Royal jelly
<i>Lact. kunkeei</i>	57.1	0.6	98.9	98.6	99.5	99.7
<i>Lact. alvei</i>	2.4	3.5	0.0	0.1	0.0	0.0
<i>Lact. floricola</i>	0.0	0.0	0.0	0.1	0.0	0.0
<i>Lact. frumenti</i>	0.0	0.0	0.0	0.1	0.0	0.0
<i>Lact. insectis</i>	28.3	60.1	0.0	0.4	0.0	0.2
<i>Lact. intestinalis</i>	0.0	0.0	0.0	0.3	0.0	0.0
<i>Lact. kefirnofaciens</i>	0.0	3.5	0.1	0.0	0.0	0.0
<i>Lact. mucosae</i>	0.0	0.0	0.0	0.0	0.2	0.0
<i>Lact. ozensis</i>	0.0	0.0	0.0	0.3	0.0	0.0
<i>Lact. plantarum</i>	0.0	0.0	0.0	0.0	0.2	0.0
<i>Lact. reuteri</i>	0.0	0.0	0.0	0.1	0.0	0.0
<i>Lactobacillus</i> sp. Bin4	0.4	0.3	0.0	0.0	0.0	0.0
<i>Lactobacillus</i> sp. Hma2	1.6	6.9	0.0	0.0	0.0	0.1
<i>Lactobacillus</i> sp. Hon2	1.4	1.3	0.0	0.0	0.2	0.0
<i>Lactobacillus</i> sp. M1T4B2	0.0	0.0	0.0	0.0	0.0	0.0
<i>Lactobacillus</i> sp. S4C	6.3	20.5	0.4	0.0	0.0	0.0
<i>Lactobacillus</i> sp. Sal8	2.6	3.4	0.6	0.0	0.0	0.0

1999) was 0.85 for IgA secretion, 0.78 for mitogen activity and 1.00 for IL-2 production.

Genotypic and phenotypic characteristics of BP104 and BP402

BP104 and BP402 were identified by BLAST analysis of approx. 1500-bp 16S rRNA sequences as sharing 100 and

99.9% identity with *Lact. kunkeei* JCM16173^T (GenBank accession number: Y11374).

Carbohydrate fermentation was compared in *Lact. kunkeei* JCM16173^T, BP104 and BP402. BP104 and BP402 fermented five of 49 tested carbohydrates (glucose, fructose, sucrose, trehalose and gluconate); *Lact. kunkeei* JCM16173^T produced acid from only glucose and sucrose (Table 3).

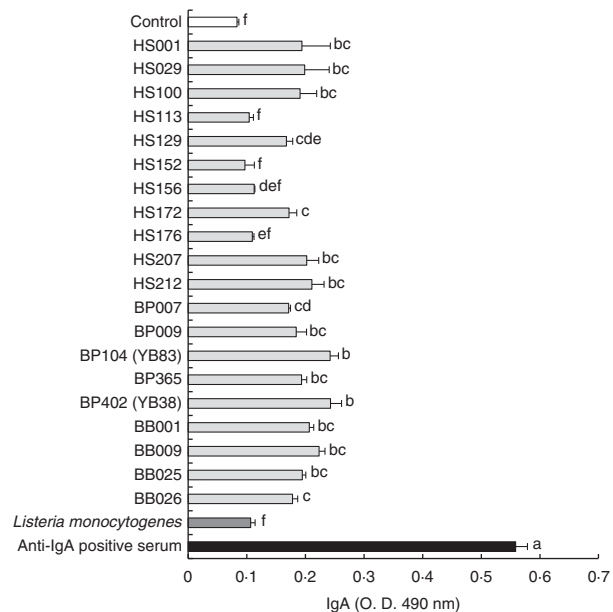


Figure 1 IgA secretion from mouse PP cells induced by *Lactobacillus kunkeei*. Each value is expressed as a mean ± standard deviation ($n = 3$). Different letters represent significant differences at $P < 0.05$ (Tukey–Kramer test). HS, honey stomach; BP, bee pollen; BB, bee bread.

RAPD-PCR analysis provided reliable differentiation of BP104, BP402 and JCM16173, each of which yielded a unique profile (Fig. 3). BP104 and BP402 were named

YB83 (deposit number: FERM BP-11438) and YB38 (deposit number: FERM BP-11439) respectively.

A pilot clinical trial of safety and IgA induction by heat-killed YB38

We performed a pilot trial of YB38 for its effect on IgA production in human subjects. During the 4-week intake of heat-killed YB38, there were no adverse effects. Table 4 shows the changes in various blood parameters. After the 4-week experiment, erythrocytes, haemoglobin, haematocrit, platelets and LDH were lower than they were before intake, although all parameters were within the normal range. No significant changes were detected in other blood parameters.

Table 5 shows the changes in saliva flow rate, salivary total protein concentration, salivary SIgA concentration, salivary SIgA secretion and salivary SIgA concentration relative to total protein concentration. Intake of heat-killed YB38 had no effect on saliva flow rate and salivary total protein concentration; however, YB38 intake was associated with a significant increase in SIgA concentration ($P = 0.001$), SIgA concentration relative to total protein concentration ($P = 0.001$) and SIgA secretion ($P = 0.020$).

Discussion

This study showed that *Lact. kunkeei* YB38 isolated from honeybee products may promote IgA production in

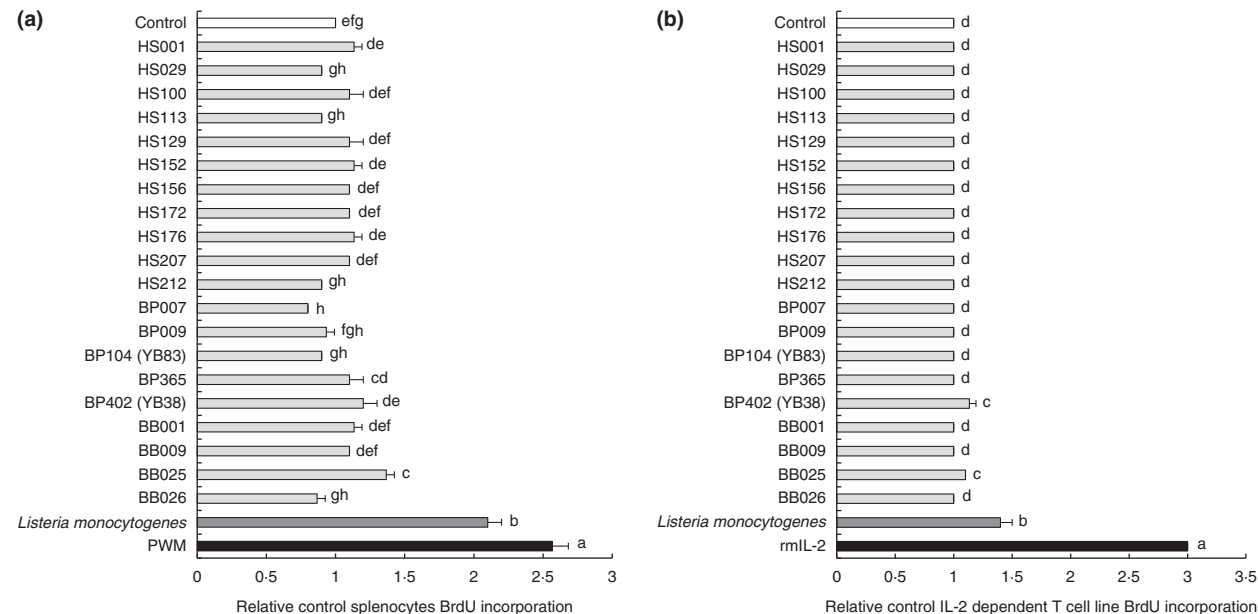
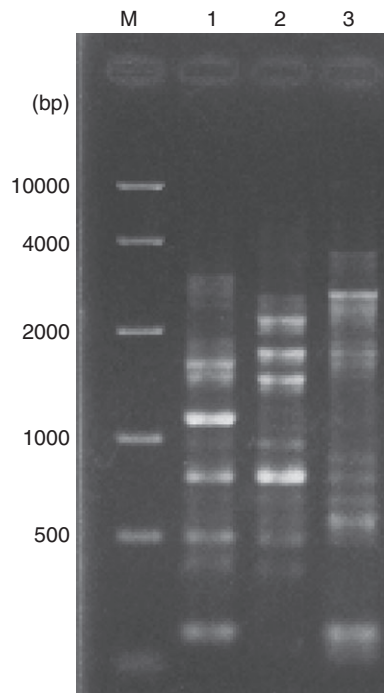


Figure 2 Mitogen activity (a) and IL-2 secretion (b) from mouse spleen cells induced by *Lactobacillus kunkeei*. Each value is expressed as a mean ± standard deviation ($n = 3$). Different letters represent significant differences at $P < 0.05$ (Tukey–Kramer test). HS, honey stomach; BP, bee pollen; BB, bee bread; PWM, Pokeweed mitogen; rmlL-2, recombinant mouse IL-2.

Table 3 Carbohydrate fermentation by *Lactobacillus kunkeei* isolates

	Glucose	Fructose	Sucrose	Trehalose	Gluconate
JCM16173 ^T	+	–	+	–	–
BP104 (YB83)	+	+	+	+	+
BP402 (YB38)	+	+	+	+	+

+, positive; –, negative.

**Figure 3** RAPD-PCR patterns obtained with primer 1254 and purified template DNA from *Lactobacillus kunkeei*. Lanes M, Marker; 1, *Lact. kunkeei* JCM16173^T; 2, BP402 (YB38); 3, BP104 (YB83).

humans. *Lact. kunkeei* was the predominant species in honey, bee pollen and bee bread, consistent with previous findings (Anderson *et al.* 2013; Corby-Harris *et al.* 2014). *Lact. kunkeei* was also the dominant species in royal jelly and other honeybee products. To our knowledge, this is the first report of microbiota analysis to be performed in royal jelly. Whole gut samples had the lowest percentage of *Lact. kunkeei*, consistent with recent findings (Corby-Harris *et al.* 2014). We assumed *Lact. kunkeei* predominance in pollen would lead to its prevalence in the gut—pollen being the principle food of the honeybee—however, the whole gut contained little of this strain, in spite of its predominance in the stomach, suggesting it does not survive passage to, or is otherwise suppressed in, the intestinal environment.

Lact. kunkeei has been isolated from honey stomach, bee pollen and bee bread (Olofsson and Vásquez 2008;

Table 4 Blood chemistry

	Before intake	After intake of YB38
Leucocyte (μl^{-1})	6052.7 \pm 969.8	5708.2 \pm 948.7
Erythrocyte ($\times 10^4 \mu\text{l}^{-1}$)	443.7 \pm 36.8	421.3 \pm 36.4**
Haemoglobin (g dl ⁻¹)	13.0 \pm 1.2	12.5 \pm 1.1**
Haematocrit (%)	41.2 \pm 2.4	38.8 \pm 2.9**
Mean cell volume (fl)	93.1 \pm 4.8	92.4 \pm 5.0
Mean cell haemoglobin (pg)	29.4 \pm 2.1	29.7 \pm 2.2
Mean cell haemoglobin concentration (%)	31.6 \pm 0.9	32.1 \pm 0.9
Blood platelet count ($\times 10^4 \mu\text{l}^{-1}$)	32.2 \pm 6.7	29.3 \pm 5.5**
Basophil (%)	0.8 \pm 0.6	0.8 \pm 0.4
Eosinophil (%)	3.2 \pm 1.8	2.5 \pm 1.3
Lymphocyte (%)	31.7 \pm 5.4	33.4 \pm 6.5
Monocyte (%)	5.1 \pm 1.0	5.1 \pm 1.5
Neutrophil (%)	59.0 \pm 6.1	58.2 \pm 6.3
Total protein (g dl ⁻¹)	7.4 \pm 0.4	7.1 \pm 0.4*
Albumin (g dl ⁻¹)	4.4 \pm 0.3	4.3 \pm 0.4
Total bilirubin (mg dl ⁻¹)	0.5 \pm 0.2	0.5 \pm 0.1
Creatinine (mg dl ⁻¹)	0.6 \pm 0.1	0.7 \pm 0.1
Creatinine kinase (U l ⁻¹)	86.1 \pm 24.5	96.0 \pm 36.1
Urea nitrogen (mg dl ⁻¹)	13.9 \pm 4.3	13.1 \pm 3.6
Uric acid (mg dl ⁻¹)	4.3 \pm 0.9	4.4 \pm 1.0
AST (U l ⁻¹)	17.8 \pm 3.9	17.2 \pm 5.1
ALT (U l ⁻¹)	14.7 \pm 4.6	14.2 \pm 5.3
LDH (U l ⁻¹)	169.5 \pm 23.4	161.2 \pm 24.7*
ALP (U l ⁻¹)	178.8 \pm 51.6	169.5 \pm 49.0
γ -GT (U l ⁻¹)	21.2 \pm 18.1	18.8 \pm 16.5
Total cholesterol (mg dl ⁻¹)	211.9 \pm 35.9	195.5 \pm 28.5
HDL cholesterol (mg dl ⁻¹)	69.5 \pm 8.3	70.4 \pm 10.3
LDL cholesterol (mg dl ⁻¹)	117.3 \pm 30.3	109.7 \pm 26.1
Triglyceride (mg dl ⁻¹)	96.4 \pm 57.0	97.6 \pm 36.1
Glucose (mg dl ⁻¹)	94.5 \pm 8.8	90.7 \pm 5.8
HbA1c (%)	4.8 \pm 0.2	4.8 \pm 0.2

AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Each value is expressed as a mean \pm standard deviation.

* $P < 0.05$, ** $P < 0.01$ before vs after intake (Student's paired *t*-test).

Vásquez and Olofsson 2009; Anderson *et al.* 2013), consistent with our findings. LAB has not been reported in royal jelly, nor was it found here. Royal jelly contains antimicrobial proteins such as royalisin (Fujiwara *et al.* 1990), and its pH is 3.5–3.9 (Takenaka and Echigo 1980), making it lethal for LAB.

Our *in vitro* screen showed that heat-killed BP104 (YB83) and BP402 (YB38) isolated from bee pollen enhanced IgA production, had little mitogenic activity and little effect on IL-2 production. *Lactobacillus* strains with mitogenic and IgA-induction activities are well known (Takeda *et al.* 1997; Kotani *et al.* 2014). The difference in YB38 and YB83 suggests interspecies or inter-strain variation in immunomodulation by *Lactobacilli*.

Table 5 Salivary parameters

	Before intake	After intake of YB38
Saliva flow rate (ml min ⁻¹)	0.43 ± 0.23	0.49 ± 0.38
Salivary total protein concentration (mg ml ⁻¹)	1.17 ± 0.32	1.22 ± 0.30
SIgA concentration (μg ml ⁻¹)	414 ± 161	660 ± 190**
SIgA secretion rate (μg min ⁻¹)	162 ± 71	320 ± 223*
SIgA concentration relative to total protein concentration (μg mg protein ⁻¹)	360 ± 135	550 ± 122**

Each value is expressed as a mean ± standard deviation. Saliva flow rate multiplied by SIgA concentration is SIgA secretion rate. SIgA concentration divided by salivary total protein concentration is SIgA concentration relative to total protein concentration.

* $P < 0.05$, ** $P < 0.01$, before vs after intake (Student's paired *t*-test).

IL-2 mRNA expression is induced in patients with Crohn's disease (Niessner and Volk 1995), so the absence of IL-2 induction by YB83 and YB38 may make them safer. The mechanism of IgA induction occurs through T cell-dependent and -independent pathways (Tezuka *et al.* 2011). Heat-killed YB38 and YB83 had little mitogenic activity in comparison with *L. monocytogenes*, which activates T cells (Kaech and Ahmed 2001) in the spleen; thus, IgA production by YB38 and YB83 in PP cells may occur via a T cell-independent pathway. However, splenic T cells may differ from the PP cells, so further experiments are needed to characterize the mechanism of IgA induction by YB38 and YB83.

The effect of LAB in the gut are not affected by metabolism and absorption, leading us to suggest the IgA responses of PP cells *in vitro* reflect what may occur after human intake. Therefore, we performed an open-label intervention study in healthy adults to investigate the safety and SIgA-promoting effects of heat-killed YB38. Erythrocyte, haemoglobin, haematocrit, platelets, total protein and LDH decreased after intake of heat-killed YB38; however, all parameters remained within the normal range, suggesting intake of heat-killed YB38 is safe. We believe the observed reduction in these factors resulted from a chance, as we were unable to replicate the findings in a replicate clinical trial of the same number of heat-killed YB38 over the same period (in-house data). Salivary SIgA secretion rate, which was significantly increased by intake of heat-killed YB38, represents the actual amount of SIgA available on the mucosal surfaces for protection against pathogens (Bishop and Gleeson 2009). The SIgA secretion rate from saliva is also the most useful clinical biomarker of upper respiratory tract infections (URTIs) (Fahlman and Engels 2005). Heat-killed YB38 may reduce the risk for URTI.

Additionally, IgA contributed to maintenance of diversified and balanced intestinal microbiota in recent report (Kawamoto *et al.* 2014), so the influence of heat-killed YB38 on IgA production may alter the intestinal microbiota. Reports of LAB-induced alterations in intestinal microbiota have relied on live LAB—few reports have examined heat-killed strains. Thus, it will be interesting to study the potential influence of heat-killed YB38 on the intestinal microbiota.

In conclusion, we confirmed that heat-killed *Lact. kunkeei* YB38 isolated from bee pollen is safe and may improve immune responses such as IgA production. However, this hypothesis requires testing in large-scale studies.

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Conflict of Interest

There are no ethical concerns or conflicts of interest with regard to this manuscript. None of the authors has any commercial associations or financial disclosures that might pose or create a conflict of interest with information presented in this article.

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