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Research Article

The Beneficial Effect of *Lactiplantibacillus Plantarum* DM083 on Restoring the Hyperglycemia in High-Sucrose Diet-Fed *Drosophila*

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Abstract

Probiotics are gaining much attention due to their beneficial functions in restoring microbial dysregulation which is the newly emerging etiology of the metabolic diseases such as hyperglycemia. The anti-hyperglycemic effects of probiotics were mostly demonstrated using rodents or human subjects, instead of *in vitro* cell lines due to the susceptibility to probiotics-derived acid compounds, leading to the ineligibility of *in vitro* cell lines for the massive screening of varied probiotic libraries. To address this issue, the current study was aimed to take advantage of the *Drosophila* to conduct the massive screening for the anti-hyperglycemic effects of 168 probiotic libraries belonging to 14 different species derived from human oral cavity. Each of 168 strains were individually treated to High-Sucrose Diet (HSD)-fed *Drosophila* and the lowest hemolymph glucose levels were measured in HSD-fed *Drosophila* supplemented with *Lactiplantibacillus plantarum* DM043, DM049, and DM083 among 168 strains. The *Lpb. plantarum* DM083 could survive at an acidic environment greater than DM043 and DM049. Heat-killed *Lpb. plantarum* DM083 failed to inhibit hyperglycemia, reflecting the importance of being alive. Importantly, *Lpb. plantarum* DM083-mediated molecular pathways related to insulin and glucose metabolisms were conserved from *Drosophila* to mammals, implying that *Drosophila*-tested *Lpb. plantarum* DM083 could be extrapolated to the rodents or humans. In conclusion, HSD-fed *Drosophila* was for the first time applied to evaluate the anti-hyperglycemic effects of 168 probiotic libraries, leading to the discovery of *Lpb. plantarum* DM083 as the best candidate which will be applicable to the rodents and humans.

Keywords: High-sucrose diet, Hyperglycemia, *Drosophila*, Massive screening, Probiotics, *Lactiplantibacillus plantarum*

Introduction

The dietary imbalance, especially High-Sucrose Diet (HSD) intake, induces chronic metabolic disorders such as hyperlipidemia and hyperglycemia [1]. Based on the hypothesis that the development of the hyperglycemia is associated with the dysregulation in intestinal microbial ecology, many scholars have tried to employ potential therapeutic probiotics to restore gut dysbiosis preclinically [2-14] or clinically [15-19], leading to the alleviation of the hyperglycemia. The probiotic strains reported to exert the anti-hyperglycemic effects belonged to various kinds of bacterial species such as *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Limosilactobacillus reuteri*, and *Limosilactobacillus fermentum*. However, most of those works lacked the description of the initial screening test for searching the target strain from varied probiotic libraries.

Due to the time and cost limitations, the rodents or human subjects are not available for a massive screening of probiotic libraries. Although being time and cost-efficient, *in vitro* cell lines are inappropriate for the probiotics due to the susceptibility to the probiotics-derived acidic compounds. Several studies made an effort to address this issue as applying the heat-killed probiotics to the *in vitro* cell lines [3,20]. However, the subsequent rodent test used the live probiotics [21], which could distort the initial screening results.

The fruit fly, *Drosophila melanogaster*, can be alternatively used for the massive screening of probiotic libraries due to the acid resistance, short life cycle, and high fecundity [22]. Importantly, HSD feeding to *Drosophila* effectively induced hyperglycemia in larva [23,24] or in adult flies [25]. In addition, the molecular mechanisms underlying hyperglycemia were shared between fly and rodent [26]. The HSD-fed *Drosophila* showed the decrease in mRNA expression of *Insulin-Like Peptide 2* (*Ilp2*), the *Drosophila* ortholog of the insulin, in Insulin-Producing Cells (IPCs) located in the brain. Similarly, the HSD-fed mice exhibited the reduction in insulin secretion from pancreatic islets [26]. The insulin deficiency induced the shortage of glucose uptake into the peripheral cells, which was accompanied by cellular fasting responses represented by the elevation of the mRNA expressions of gluconeogenic genes such as *phosphoenolpyruvate carboxykinase* (*Pepck*) and *glucose 6-phosphatase* (*G6Pase*) [23-25,27]. In particular, *Drosophila* contains *trehalose 6-phosphatase* (*Tps1*) in addition to *G6Pase* for gluconeogenesis [28].

Human oral cavity is colonized with a variety of intrinsic probiotics. The aim of this study is to take advantage of HSD-fed *Drosophila* to search out a new anti-hyperglycemic probiotic strain among 168 probiotic libraries derived from human Tongue Coating (TC) biospecimens donated from 120 healthy volunteers (100 adults and 20 younger children).

Materials and Methods

Isolation and identification of TC-originating probiotic strains

All procedures were conducted in accordance with relevant guidelines and regulations approved by the ethics committee of the Institutional Review Board of the Apple Tree Dental Hospital (approval number: ATDH-2021-0001) and the Korea National Institute for Bioethics Policy (approval number: P01-202111-31-002). The TC samples, collected from 100 adults and 20 younger children, were distributed by the Biobank of Apple Tree Dental Hospital, a part of the Korea Biobank Network (KBN). The TC samples were resuspended in 3 mL of phosphate-buffered saline (PBS) and stored at -80 ˚C until used. To isolate TC-derived probiotic strains, 1 mL of each TC sample was plated onto ten plates (100 mL per plate) of LactoBacillus Selective (LBS) agar (Kisan Bio Inc., Korea) and anaerobically incubated for 48 h at 37 ˚C. The colonized isolates were identified by 16S rRNA partial sequencing (Macrogen Inc., Korea).

Fly culture and food

The *Drosophila melanogaster* white-eyed w^{1118} line was kept at a density of fifteen males and five females in each vial with 12 hour on/off light cycle at 25 ˚C. Three-day old flies fed *ad libitum* were used in all experiments. Flies were raised on food containing 2% (w/V) yeast and three different amounts of sucrose (w/V) (10%, 20%, and 40%), indicated with S10Y2, S20Y2, and S40Y2, respectively. The starvation diet contains only 1% sucrose.

Probiotics supplementation and hemolymph glucose measurements

Three-day old flies were transferred to vials containing the starvation diet and maintained for 12 h. Fasted flies were refed the experimental foods supplemented with probiotics at a concentration of 10^{10} Colony-Forming Units (CFU) per mL. After three days, males were anesthetized with posterior abdomen cropped, and vortexed in phosphate-buffered saline (PBS), allowing hemolymph to be eluted. After centrifuging at 9,000 g for 5 minutes at 4 ˚C, supernatants were used for determination of glucose levels by using the glucose detection kit (Sigma Aldrich Inc., USA). At least three biological replicates were measured on a microplate reader (SpectraMax iD3, Molecular Devices Inc., USA).

Acid-resistance test of probiotics

Resistance to low pH was evaluated as described by Ye et al. [28] with minor modifications. Briefly, probiotic strains were anaerobically cultured in de Man Rogosa Sharp (MRS, Sigma-Aldrich Inc., USA) liquid for 48 h at 37 ˚C. The cultures were inoculated into PBS adjusted to pH 2.5 or pH 6.5. After 6 h, the cultures were ten-fold diluted four times, thereafter each 100 µL of $1^{st} \sim 4^{th}$ dilutions were cultured on MRS agar. The x number of CFU was counted in y times diluted plate which presented the number of CFU between 30 and 200. The survival rate was calculated as the ratio of CFU of pH 2.5 to that of pH 6.5. The experiments were performed in triplicate and mean values were calculated.

Quantitative RT-PCR

Total mRNA was extracted using the RNeasy mini kit (Qiagen Inc., USA). The cDNA was generated with 0.5 µg total mRNA using the PrimeScript™ RT-PCR kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. The generated cDNA was used for real-time RT-PCR using Exicycler™ 96 Real-Time PCR systems (Bioneer Inc., Korea), SYBR Green PCR master mix (Thermo Fisher Scientific, USA), and primers for *Ilp2* (5-CTGAGTATGGTGTGCGAGGA-3 and 5-CAGCCAGGGAATT-

GAGTACAC-3), *CCHa2* (5-TCGTTATCTGCACCGTGGTC-3 and 5-CCCTTTTTCGCTTGGCTCT-3), *Pepck* (5-AGAAGAAG-TACATCACTGCCGCCT-3 and 5-TCCCTGCGAGTCAAACT-TCATCCA-3), *Tps1* (5-TCCGATGAGATCCTACAGGGTATG-3 and 5-CGCCATGTTCCACCAGCAGATTG-3), and *Rp49* (5-CG-GATCGATATGCTAAGCTGT-3 and 5-GCGCTTGTTCGATC-CGTA-3). The reaction conditions consist of the first denaturation step for 3 min at 95 ˚C followed by 40 cycles of a denaturation step for 10 s at 95 ˚C and an annealing/extension step for 20 s at 60 ˚C.

Antibiotic susceptibility test

Minimal Inhibitory Concentration (MIC) values for probiotics were determined in LSM medium (90% of Iso-Sensitest broth (KisanBio Inc., Korea) and 10% MRS broth) according to the ISO 10932:2010 broth microdilution procedure [30]. LSM medium was supplemented with serial dilutions of antibiotic compounds including GEN (0.5–256 mg/L), KAN (2–1,024 mg/L), STR (0.5–256 mg/L), TET (2–1,024 mg/L), ERY (0.016–8 mg/L), CLIN (0.032–16 mg/L), CHL (0.125–64 mg/L), and AMP (0.032–16 mg/L). Overnight-cultures of probiotics were inoculated into the LSM medium containing antibiotics and anaerobically incubated at 37 ˚C for 2 days. MIC was determined as the lowest concentration of antimicrobial compounds at which the growth of the probiotics was inhibited. The growth was measured at 600 nm absorbance using a SpectraMax iD3 microplate reader (Molecular Devices, USA) and MICs were compared to the cut-off values recommended by the European Food Safety Authority (EFSA) [31].

Hemolysis test

Hemolysis was observed by anaerobic incubation of the probiotics in Tryptic Soy Agar (TSA) (Fisher Scientific Inc., USA) supplemented with 5% sheep blood (KisanBio Inc., Korea) at 37 ˚C for 2 days [32]. The loss of blood color around colonies indicates hemolysis.

Statistical analysis

All data were expressed as mean ± Standard Deviation (SD). Statistical analysis was performed through the analysis of variance (ANOVA) followed by the Tuckey's multiple comparison. Statistical significance was attributed to p values < 0.05 (*), 0.01 (**), and 0.001 (***). The software GraphPad Prism v5 (GraphPad Inc., USA) was used for the analysis.

Results

Isolation of probiotic strains from the human TC biospecimens

The human TC specimens distributed by the Korea Oral Biobank Network (KOBN) were originated from 100 adults (aged from 19 to 80) and 20 younger children (aged under 9). As the results of cultivating each TC specimen on LBS agar plates, a total of 168 colonies were isolated, which belonged to 9 different genera and 14 different species according to the reclassified nomenclature (Table 1) [33]. *Lacticaseibacillus rhamnosus* (n = 52) showed the highest incidence, followed by *Limosilactobacillus fermentum* (n = 32), *Lactiplantibacillus plantarum* (n = 15), *Lactobacillus gasseri* (n = 14), *Latilactobacillus curvatus* (n = 14), *Limosilactobacillus vaginalis* (n = 13), *Latilactobacillus sakei* (n = 8), *Lacticaseibacillus paracasei* (n = 7), *Ligilactobacillus salivarius* (n = 6), *Limosilactobacillus mucosae* (n = 2), *Lentilactobacillus sunkii* (n = 2), *Levilactobacillus brevis* (n = 1), *Levilactobacillus graminis* (n = 1), and *Liquorilactobacillus nagelii* (n = 1). Among them, *Lcb. rhamnosus*, *Llb. fermentum*, *Lpb. plantarum*, *Llb. sakei*, and *Lcb. paracasei* are species found in both adults and younger children.

No.	Species name	Adults	Younger children	Sum
1	*Latilactobacillus curvatus*		14	14
2	*Latilactobacillus graminis*		1	1
3	*Latilactobacillus sakei*	1	7	8
4	*Lacticaseibacillus rhamnosus*	49	3	52
5	*Lacticaseibacillus paracasei*	5	2	7
6	*Lactiplantibacillus plantarum*	12	3	15
7	*Limosilactobacillus fermentum*	23	9	32
8	*Limosilactobacillus vaginalis*	13		13
9	*Limosilactobacillus mucosae*	2		2
10	*Lactobacillus gasseri*	14		14
11	*Ligilactobacillus salivarius*	6		6
12	*Lentilactobacillus sunkii*	2		2
13	*Levilactobacillus brevis*	1		1
14	*Liquorilactobacillus nagelii*	1		1

	Sum	129	39	168

Table 1: The list of probiotic strains isolated from adults and younger children.

Induction of hyperglycemia in *Drosophila*

To find out the sucrose concentration to induce hyperglycemia in *Drosophila*, adult flies were fed three kinds of diets differing in sucrose concentrations (10%, 20%, and 40%, respectively). As the results, the hemolymph glucose levels increased in proportion to the sucrose concentrations, leading to peak glucose level at 40% sucrose (Figure 1).

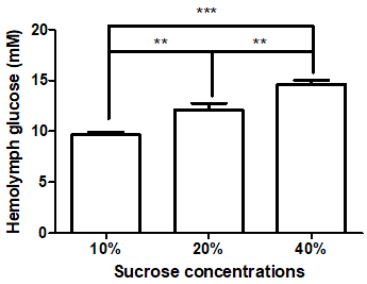

Figure 1: The graphs showing hemolymph glucose levels measured in *Drosophila* adult flies fed 2% yeast and three different concentrations of sucrose as indicated. Three times (five males per time) were replicated and subjected to ANOVA followed by the Tuckey's multiple comparison. Statistical significance was attributed to p values < 0.01 (**) and 0.001 (***).

The massive screening of probiotic libraries using *Drosophila*

The massive screening was conducted to examine the anti-hyperglycemic effects of 168 libraries using *Drosophila* adult flies fed 40% sucrose and 2% yeast diet (triplicate of five males for each probiotic strain). As the results, three strains belonging to *Lpb. plantarum* species (DM043, DM049, and DM083) were determined to be the most effective in suppressing hyperglycemia as indicated with gray bars in Figure 2.

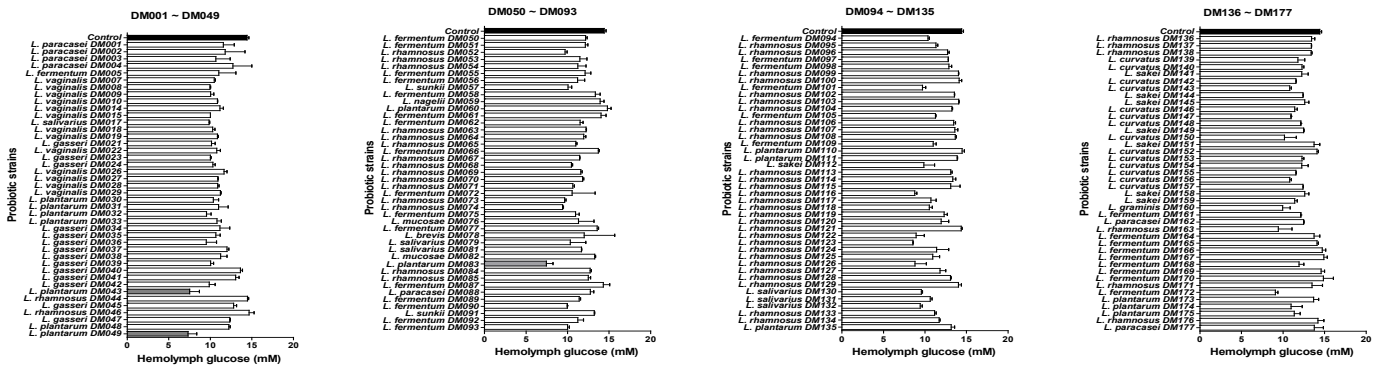

Figure 2: The graphs showing hemolymph glucose levels of *Drosophila* fed 40% sucrose and 2% yeast (S40Y2, black bars) and S40Y2-fed *Drosophila* supplemented with 168 probiotic libraries from DM001 to DM177 with exemption of nine. Gray bars indicated the probiotic strains exerting the strongest effects on anti-hyperglycemia.

Acid-tolerance test

The acid-tolerance test determined that *Lpb. plantarum* DM083 was significantly more acid-tolerant than DM043 or DM049 (Figure 3A), implying that the *Lpb. plantarum* DM083 is more likely to remain alive after passing through the stomach compared to DM043 or DM049. The significance of being alive in relation to the anti-hyperglycemic effect of *Lpb. plantarum* DM083 was reflected by the result that the heat-killed *Lpb. plantarum* DM083 failed to restore hyperglycemia (Figure 3B).

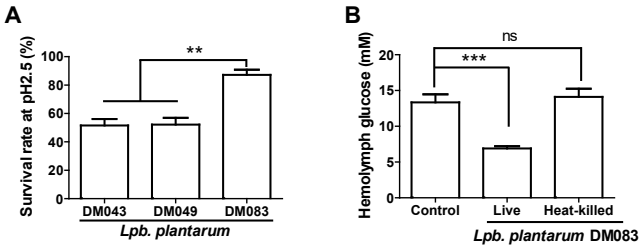

Figure 3: (A) The graphs illustrating survival rates (%) of *Lpb. plantarum* DM043, DM049, and DM083 which were calculated as the ratio of the number of CFU incubated in pH2.5 to that incubated in pH6.5. (B) The graphs demonstrating hemolymph glucose levels of S40Y2-fed *Drosophila* supplemented with live or heat-killed *Lpb. plantarum* DM083. Three times were replicated and subjected to ANOVA followed by the Tuckey's multiple comparison. Statistical significance was attributed to p values < 0.01 (**) and 0.001 (***).

The mechanisms underlying the anti-hyperglycemic effect of probiotics

To investigate the molecular mechanisms under which *Lpb. plantarum* DM083 can alleviate the hyperglycemia, mRNA levels of genes related to the insulin and glucose metabolisms were measured in *Drosophila* adult flies feeding 10% sucrose (normal diet, ND), 40% sucrose (high-sucrose diet, HSD), and HSD + 10^{10} CFU/mL *Lpb. plantarum* DM083 (HSD+DM083). The mRNA levels of *CCHamide-2* (*CCHa2*), the insulin secretagogue, measured in the intestine tissues were lowered in HSD compared to ND, but significantly upregulated in HSD+DM083 (Figure 4A). The mRNA levels of *Ilp2*, the *Drosophila* ortholog of insulin, measured in the brain tissues were also reduced in HSD compared to ND, but significantly increased in HSD+DM083 (Figure 4B). The mRNA levels of both *Pepck* and *Trehalose-6-phosphatase* (*Tps1*), the gluconeogenic genes, measured in abdomen tissues were higher in HSD compared to ND, but significantly downregulated in HSD+DM083 (Figure 4C and D). Taken together, *Lpb. plantarum* DM083 intervention successfully restored HSD-induced dysregulations in insulin and glucose metabolisms.

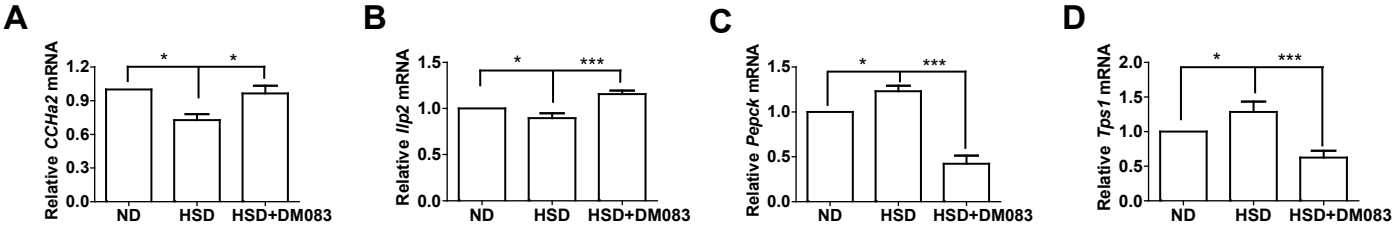

Figure 4: The graphs showing mRNA levels of *CCHa2* (A), *Ilp2* (B), *Pepck* (C), and *Tps1* (D) in *Drosophila* fed ND, HSD, and HSD+DM083. Three times were replicated and subjected to ANOVA followed by the Tuckey's multiple comparison. Statistical significance was attributed to p values < 0.05 (*) and 0.001 (***).

Safety evaluations of *Lpb. plantarum* DM083

The antibiotic susceptibility of the *Lpb. plantarum* DM083 was evaluated by MIC values according to EFSA criteria for *Lpb. plantarum*, resulting that *Lpb. plantarum* DM083 was susceptible to all the tested antibiotics as indicated in Table 2. Additionally, no hemolytic activity of the *Lpb. plantarum* DM083 was observed when cultured on TSA supplemented with sheep blood for 2 days at 37 ˚C (data not shown).

Class	Aminoglycoside		Tetracycline	Macrolide	Lincomycin	Amphenicol	β-Lactam
Antibiotic (mg/L)	GEN	KAN	TET	ERY	CLIN	CHL	AMP
Cut-off value*	16	64	32	1	2	8	1
DM083	2	16	32	0.5	0.5	8	0.5
*Based on EFSA criteria for *Lpb. plantarum*.							

Table 2: Antibiotics susceptibility test of *Lpb. plantarum* DM083.

Discussion

Lpb. plantarum is a non-gas-producing lactic acid bacterium that is generally regarded as safe (GRAS) [34]. The representative strain for the *Lpb. plantarum* is WCFS1 isolated from saliva [35], and most of other strains belonging to the *Lpb. plantarum* were isolated in fermented foods [34] and infant faces [36]. Interestingly, plenty of research reports exhibited the anti-hyperglycemic effects exerted by various kinds of the *Lpb. plantarum* strains such as CCFM0236 [2], Ln4 [3], MTCC5690 [4], NCDC17 [10], 299v [37], 13 [38], TN627 [39], DSM15313 [40], OLL2712 [41], NCIMB8826 [42], MG4296 [43], SCS2 [44], SS18-5 [45], LRCC5310 [46], Y15 [47], Dad-13 [48], SHY130 [49], and NC8 [50]. Especially, *Lpb. plantarum* HAC01 was intensively investigated to show the anti-hyperglycemic effects in the high-fat diet (HFD)/streptozotocin (STZ)-treated mice [12] and humans [18]. Those numerous references implies that *Lpb. plantarum*-belonged strains share the crucial factors being advantageous over other probiotic species in modulating the host glucose and insulin metabolisms although the concrete evidence has remained obscure. Importantly, the application of *Drosophila* adult flies to probiotic libraries including 14 species and 168 strains also demonstrated that the anti-hyperglycemic effects of *Lpb. plantarum*-belonged strains (DM043, DM049, and DM083) are superior to other probiotics, contributing to the reinforcement of those references above.

The mechanisms underlying the probiotic effects can be attributed to the interaction between probiotics-derived metabolic compounds and gastrointestinal tract. Several *Lpb. plantarum*-derived compounds such as phenyllactic acid [51], butyrate [52], gamma-aminobutyric acid (GABA) [53], and exopolysaccharide [54] have been already specified to cause the anti-hyperglycemic effects. Those microbial products can interact with the gastrointestinal tract of which enteroendocrine cells play the pivotal roles to secrete various types of peptide hormones. For instance, the microbial GABA was reported to increase Glucagon-Like Protein-1 (GLP-1) secretion in enteroendocrine cells [55]. GLP-1 is released from L-cells in mammalian small intestine and relayed to the pancreatic b-cells, contributing to enhance the insulin secretion and thus rescue diabetic hyperglycemia and hyperlipidemia [56].

It is plausible that the anti-hyperglycemic effect of the *Lpb. plantarum* DM083 also might be derived from its bioactive molecules. The *Lpb. plantarum* DM083 genome (GenBank CP099962.1) possesses the glutamate decarboxylase (NHN79_14330) [57] which catalyzes GABA production, implying that the *Lpb. plantarum* DM083 could contribute to the stimulation of GLP-1 release from enteroendocrine cells and thereafter insulin secretion from pancreas [58]. The current study demonstrated that *Lpb. plantarum* DM083 increased the intestinal mRNA levels of *CCHa2* and the brain mRNA levels of *Ilp2*. On the behalf of GLP-1, in *Drosophila*, enteroendocrine cell-specific hormone CCHa2 is relayed to the IPCs located in the brain, contributing to the elevation of the mRNA level of the *Ilp2*, the *Drosophila* ortholog of the insulin [59,60]. It should be needed to examine if the *Lpb. plantarum* DM083 can produce GABA in the further study.

Conclusions

The significance of the current finding is that the massive screening of probiotic libraries can be highly available using the acid-resistant, time- and cost-efficient model of *Drosophila* of which molecular mechanisms underlying glucose and insulin metabolisms are conserved from flies to mammals. It is noteworthy to propose that the anti-hyperglycemic effect of the *Lpb. plantarum* DM083 demonstrated by HSD-fed *Drosophila* would be also remarkable in the diabetic rodents or humans.

Author contributions

D.-Y.P. developed the project, designed experiments, evaluated the data, and drafted the manuscript. J.-H.L. and Y.-J.K. conducted *Drosophila* experiments. J.H. conducted biospecimen collection and microbial experiments. Y.-Y.K. and H.-S.K. provided clinical resources and critical comments. D.-Y.P. supervised the project and edited the final version of the manuscript. All authors discussed drafts and approved the final manuscript for publication.

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Ethical Guidelines

The collection and distribution of biospecimens by the Biobank of Apple Tree Dental Hospital was approved by the ethics committee of the Apple Tree Medical Foundation (IRB number: ATDH-2021-0001). All participants understood the purpose of the study and provided informed consent. The study using the distributed TC biospecimens (IRB approval number: P01-202111-31-002) was approved by the ethics committee of the Public Institutional Review Board (http://public.irb.or.kr) run by the Korea National Institute for Bioethics Policy.

Conflict of Interest

D.-Y.P., J.-H.L., Y.-J.K., and J.H. are employees of DOSCMEDI OralBiome. Y.-Y.K. and H.-S.K. are employees of the Apple Tree Medical Foundation and own DOSCMEDI OralBiome stock.

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