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A novel study on the identification and sleep inducing effect of plant lactic acid bacteria (*Lactobacillus helveticus* KJMA-0001) isolated from Jeju Aloe vera (*Aloe Barbadensis* Miller)

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Abstract: Lactic acid bacteria (LAB) are beneficial bacteria for humans and animals. However, the sleep characteristics and functions of plant LAB in human remain unclear. Here, we isolated LAB from the jeju*Aloe vera* in Korea, and identified one *Lactobacillus helveticus* KJMA-0001(Alab™) using matrix associated laser desorption/ionization time of flight and 16S rRNA/DNA full genome sequence analyses. Alab™ survived at pH 4.5for 24 h. In addition, this strain survived well in simulated gastric juice of humans containing pepsin and exhibited high resistance to bile salts.
Effect of Alab™ on sleep related behaviors and patterns in *Drosophila melanogaster* was examined in both baseline and caffeine treated conditions. Total activities of flies significantly decreased in 20 mg/mL Alab™ during nighttime or daytime compared with the control. This Alab™ mediated effect was partly observed in caffeine-treated flies. Alab™ up regulated mRNA expressions of gamma-amino butyric acid (GABA) receptors and serotonin receptor, and GABA receptors were more strongly regulated than serotonin receptor. This study demonstrates that aAlab™ improves sleep related behaviors, including sleeping time, by modulating GABAergic/serotonergic signaling.
This Alab™ can be utilized as a novel probiotic in the sleep related drugs and healthfoods of beneficial LAB.

Keywords: *Aloe vera*; plant lactic acid bacteria, *Lactobacillus helveticus*, sleep inducing effect, gamma-amino butyric acid activity, Alab™

I. INTRODUCTOION

Lactic acid bacteria (LAB) are beneficial microorganisms found in humans, insects, and animals [1]. LAB fermentation plays an important role in the food industry, particularly for dairy products. Strains of LAB are generally recognized as safe (GRAS) food grade microorganisms and used as probiotics to benefit human health [2]. Among plant derived LAB, symbiotic LAB of Kimch and pickles have been studied extensively [3-5]. However, this is the first time to study the symbiotic relationship between LAB in Jeju *Aloe vera* in Korea, isolate and identify LAB from aloe, and study sleep functionality.

Nagpal R. and his colleagues [6] showed that *Aloe vera* extract can promote the growth of probiotic *Lactobacillus* strains at certain concentrations and can be used as prebiotics for manufacturing symbiotic therapeutic products. The use of aloe extract in probiotic foods could be a promising trend in the use of herbs as well as functional ingredients in healthfoods [7]. *Aloe vera* fortified probiotic products was prepared and effect of storage on syneresis, pH, *Lactobacillus* spp. count, and *Bifidobacterium*spp.count of *Aloe vera* fortified probiotic product was assessed for storage study [8-10]. Taverniti V and Guglielmentti S. [11] showed that *Lactobacillus helveticus*had a number of health promoting properties in interventional studies and clinical trials.

A novel *Lactobacillus helveticus*KJMA-0001 (Alab™) strain (pH 4.5-5.5) isolated from the surface of *Aloe vera* leaves was cultured at room temperature for 2-3 days and then identified through RAPD analysis and comparison of 16 rRNA sequences. A strain called Alab™ derived from *Aloe vera* leaves expressed high levels of the glutamate carboxylase gene, which produces the beneficial neurotransmitter r-aminobutyric acid (GABA). Kim DM. and his colleagues' patent suggests that the new *Lactobacillus helveticus* KJMA-0001 strain Alab™ could be a resource for GABA production [12-14].

Sleep, occupying one-third of human life, is one of the most important natural states to maintain good health and wellbeing in life [15]. Sleep disorders cause noticeable impairments in daytime function or behavior

and are accompanied by various problems, such as tiredness, memory problems, accident proneness, lethargy, and other physical or mental impairments [16]. It frequently causes clinical problems regardless of gender and age [17]. Sleep disorder patients are prone to depression, anxiety and alcohol dependence [18] Insomnia, one sleep disorder, is characterized by difficulty falling and/or staying asleep despite a sufficient opportunity to sleep [18]. Adult disease such as cardiovascular disease and obesity was shown to be implicated to insomnia [19]. Management of insomnia has usually been achieved through treatment with pharmacological agents, including benzodiazepine, antidepressants, barbiturates, and anti-psychotics [19]. However, these medications have shown negative side effects, such as daytime sedation, hangover, and drug dependence [20]. Accordingly, many people seek solutions to sleep disorders *via* natural substances and dietary supplements [20].

Several herbal materials, such as Valerian (*Valeriana offcinalis*), Hops (*Humulus lupulus* L.), and chamomile (*Matricaria chamomilla*), have been known to be effective in sleep disorders and insomnia [21, 22], but there are no studies on the sleep improvement effect of aloe derived LAB (Alab^{TM}).

The gamma-aminobutyric acid (GABA) receptors are a kind of receptor that responds to a neurotransmitter called GABA, the major inhibitory compound in the central and peripheral nervous systems (C/PNS) [23]. GABA receptors have been known to affect cognition, including sleep and wakefulness, by coordinating with glutamatergic processes [24]. These receptors are divided into two classes, GABAA and GABA B, according to their rate of response to GABA [19]. GABA A receptors have been widely studied as a target site for CNS sedative herbs and many pharmacological medicines [21]. *Drosophila melanogaster*, the fruit fly, has been used as an invertebrate model in many studies. Invertebrates such as *D. melanogaster* share a similar sleep regulatory mechanism associated with a circadian rest activity cycle and homeostatic processes with vertebrate systems [25]. In particular, sleep patterns of *D. melanogaster*, unlike vertebrates, are evaluated by multiple factors such as behavior, activity, and electrophysiology [15, 26], but fly sleep is modulated by some stimulants and hypnotics that also affect human sleep [15].

In this study, we evaluated the sleep-promoting effects of a Alab^{TM} using fruit flies. Although several studies have investigated the sleep related effects of herbal materials [27, 28], systematic research on the sleep-promoting effects of Alab^{TM} has been limited. The current study describes the effects of the Alab^{TM} through the analysis of multiple sleep episodes in a hitherto unstudied *Drosophila* model.

II. MATERIALS AND METHODS

2.1. Plant source and Isolation of Alab^{TM}

Aloe vera, *Aloe Barbadensis* Miller, were collected from KimJungMun Aloe fields in Jeju, South Korea. 150 *Aloe vera* seedlings were rinsed twice with sterile water and their leaves were carefully removed and collected in 10 mM phosphate buffer, pH 7.0 (PB). The leaves were homogenized with a plastic pestle and serially diluted with PB. The diluted solution was spread onto de Man, Rogosa, and Sharpe (MRS) agar medium (Difco, Detroit, MI, USA) and incubated at 37˚C for 3 days in an incubator (Thermo Scientific, Waltham, MA, USA). Bacterial colonies that were uniform, round, and white were selected, purified, and stored at -80˚C in MRS broth mixed with glycerol (25%, v/v)**.**

2.2. Identification of Alab^{TM}

To confirm the identity of bacterial isolates, whole cell matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry using the program MALDI Biotyper v.3.0 (Bruker Daltonics, Bremen, Germany) were performed according to the manufacturer's instructions (Bruker Daltonics). Bacterial samples for MALDI-TOF MS were prepared as previously described [29]. Mass spectra were analyzed using a micro flex LT mass spectrometer (Bruker Daltonics) and default parameters (positive linear mode; laser frequency, 60 Hz; ion source 1.0 voltage, 20 kV; ion source 2voltage, 16.7 kV; lens voltage, 7.0 kV; and mass range, 2 kDa to 20 kDa). For each spectrum, 240 laser shots in 40shot steps from different positions of the sample spot were accumulated and analyzed (automatic mode, default settings) as previously described [29]. MALDI-TOF MS data were interpreted according to the manufacturer's instructions (Bruker Daltonics). Scores of 2.0 are considered reliable for the species level, and scores of 1.7 but <2.0 are acceptable for the genus level, and scores below 1.7 are considered unreliable [29].

The partial 16S rRNA region was amplified with the primers 27mF (50-AGAGTTTGATCMT GGCTCAG-30) and 1492mR (50-GGYTACCTTGTTACGACTT-30) [30]. Total DNA extraction was conducted as described by Kim *et al.* [12-14]. Polymerase chain reaction (PCR) was performed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using PCR premix (Bioneer, Daejeon, Korea), 10 ng genomic DNA, and 1 mM of each primer with a program of 98˚C for 30 s, 55˚C for 30 s, and 70˚C for 1 min, followed by a final 4min extension at 72˚C. The PCR product was confirmed using 0.8% agarose gel electrophoresis and purified using the Expin Gel SV kit (Gene All Biotechnology, Seoul, Korea). Sequencing was performed by Macrogen's sequencing service (Macrogen, Daejeon, Korea). The resulting DNA sequences were analyzed using the National Center for Biotechnology Information GenBank database (https://www.ncbi.nlm.nih.gov/blast/). A phylogenetic tree based on the nucleotide sequences of the 16S rRNA

gene was generated using MEGA 7.0 software and the neighbor joining method [31]. *Lactobacillus helveticus* KJMA-0001 strain Alab™ was used as an outgroup because it is phylogenetically closely related to the genera *Lactococcus* [32].

2.3. Survival of Alab™ at acidic pH

Survival of Alab™ strains at weak acidic pH was tested. MRS broth was adjusted to pH 4.5 or 5.5 using 1.0 N NaOH. MRS broth adjusted to pH 7 was used as a control. Cells from 2days old cultures of the one isolate grown at 37˚C were inoculated into adjusted MRS broth at a concentration of approximately 10^9colonyforming units (CFU)/mL and incubated at 37˚C for 24hrs. Samples were taken at 6 hrs intervals, serially diluted, and spread onto MRS medium. The plates were incubated at 37˚C for 3 days. The viable cell population was determined by colony counting.

2.4. Survival of Alab™ in simulated gastric juice and bile salt solution of humans

Simulated gastric juice of humans was prepared by adding 1% pepsin to MRS broth adjusted to pH 2.2 or 2.5 using 1.0 N HCl, according to a previously reported method [4]. Cells from 2days old cultures of the three isolates grown at 37˚C were inoculated into simulated gastric juices at a concentration of approximately 10^9 colony forming units (CFU)/mL and incubated at 37˚C for 2 hrs. Samples were taken at 30 min intervals, serially diluted, and spread onto MRS medium. The plates were incubated at 37˚C for 3 days. The viable cell population was determined by colony counting.

For the bile tolerance assay, cells from 2days old cultures of the three isolates grown at 37˚C were inoculated into MRS broth containing 0.1 or 0.5% (w/v) bile salt (oxgall) (Difco) at a concentration of approximately 10^9 CFU/mL and then incubated at 37˚C for 24 hrs according to a previously reported method [5]. Samples were taken at 6 hrs intervals, serially diluted, and spread onto MRS medium. The plates were incubated at 37˚C for 3 days. The viable cell population was determined through colony counting.

2.5. Fly Stocks

Wildtype *D. melanogaster* Canton-JE strain flies were obtained from the Drosophila Stock Center at Jeju National University. The flies were maintained in standard fly bottles containing sucrose medium (sucrose, cornmeal, dried yeast, agar, propionic acid, and p-hydroxy benzoic acid methyl ester solution) and raised under a 12 : 12 h light : dark cycle at 25 ± 1°C in 60% relative humidity (RH). Alab™ samples were added to sucrose medium with the indicated concentrations. Prior to sample treatment, 2–5days old male flies were collected under anesthesia using CO_2.

2.6. Behavioral Assays

Alab™ samples were dissolved in distilled water and mixed in sucrose agar media (5% sucrose and 1% agar) for the locomotor activity assays. Single treatments of Alab™ included 2, 5, 10, and 20 mg/mL concentrations. In the *Drosophila* Activity Monitoring system (DAM; Tri Kinetics, Waltham, MA, U.S.A.), flies were kept in individual glass tubes for analysis of behavior patterns in each fly. Group activity of flies for single treatments of Alab™ group was assessed by the Locomotor Activity Monitoring system (LAM, Tri Kinetics) to provide measures of locomotor activity combined with social behaviors. All the experiments were triplicated (DAM: 10 flies per replicate, LAM: 30 flies per replicate). Flies were subjected to a 24 hrs adaptation period in the tubes, and all activities were then recorded every 1 or 30 min for 4–7 days under constant darkness (DD) at 25 ± 1°C. A 0.1% caffeine solution (10 mg/mL) was used as a stimulant in the awake condition [33]. Data were generated by DAM management software (Tri Kinetics) with controls for environmental stimuli, such as sound and light. The number of infrared detector interruptions at each time interval was recorded and visualized using Actogram J software. Sleep analyses were performed during dark hours of the daily cycle in the control group and caffeine induced awake groups and compared. Sleep parameters were calculated by summing up all the activity counts recorded in the 12 hrs dark period (nighttime). Dark phase activity was calculated by summing total activity, and total dark phase sleep was calculated by summing the duration of sleep. Sleep was defined as periods of uninterrupted behavioral immobility and inactivity longer than 5 min (0 counts per min) [34]. In addition, the number of sleep episodes were counted and summed [33].

2.7. GABA A-Benzodiazepine Receptor Binding Assay

The GABAA receptor binding assay was performed with modification according to the method described by Risa *et al*. [35] and Kahnberg *et al* [36]. The cerebral cortex of male four Sprague Dawley rats was homogenized for 10 s in 20 mL of Tris–HCl buffer (30 mM, pH 7.4, 0–4°C). The suspension was centrifuged at 0–4°C for 15 min at 27,000×g, and the pellet was washed three times with Tri–HCl buffer. The washed pellet was resuspended in 20 mL of Tris–HCl buffer, after which the suspension was incubated in a water bath at 37°C for 30 min, followed by centrifugation at 10 min at 27,000×g. The final pellet was suspended in 30 mL of Tris–HCl buffer (50 mM, pH 7.4) and stored in aliquots at −80°C until assayed. The final suspension (membrane

suspension) was adjusted with the concentration of 30.5 µg protein in 100 µL Tris-citrate buffer (50 mM, pH 7.1, 0–4°C) to be used for the binding assay. The membrane suspension (300 µL) was added to 25 µL test solution and 21 µL of [3H]-flumazenil and incubated on ice for 40 min for the binding, in which its final concentration is 0.8 nM. Samples used to GABAA receptor binding assay were Alab™ (200 mg/mL). The binding was terminated by filtration onto a Whatman GF/B glass fiber filter using a harvester (Brandel Inc., Gaithersburg, MD, U.S.A.) with ice-cold 30 mM Tris–HCl buffer to remove unbound [3H]-flumazenil. The bound samples were counted with 5 mL of liquid scintillation cocktail solution (Aqualight Beta, Hidex Personal Life Science, Turku, Finland) in bottle using a Hidex 300SL counter (Hidex, Turku, Finland). Total and nonspecific bindings were determined using the binding buffer and benzodiazepine (1.0 µM, final concentration), respectively. The displacement percent of radioligand binding was determined by the following equation (DPM: disintegrations per minute, TB: total binding, and NSB: nonspecific binding).

$$\text{Binding displacement (\%)} = [1 - (\text{DPM}-\text{DPM}_{NSB}) / (\text{DPM}_{TB}-\text{DPM}_{NSB})] \times 100$$

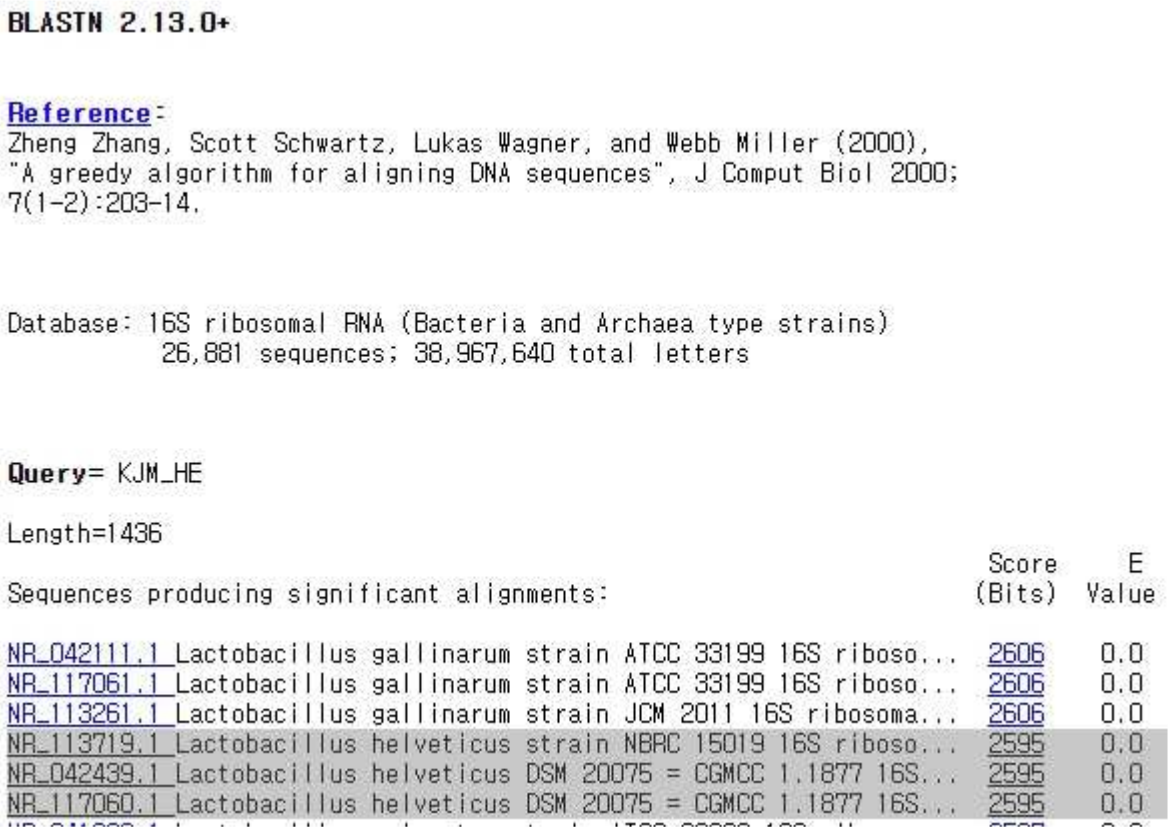
2.8. Statistical Analyses

All statistical analyses were performed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL, U.S.A.). Differences between groups were evaluated by one way ANOVA and Tukey's multiple comparison tests. Statistic values of $p<0.05$ were considered significant. All data are reported as means ±standard error of the means (S.E.M.). Student's t-tests were also used to analyze differences.

III. RESULTS

3.1. Identification of Alab™ in the Jeju Aloe

To isolate the Alab™ associated with the aloe, bacteria were isolated from the seedlings of healthy *Aloe vera*. Gram positive, oxidase negative bacteria with round, white colonies were consistently recovered on MRS medium containing 2% glucose. One bacterial isolate (*Lactobacillus helveticus*KJMA-0001, Alab™) was selected for identification and characterization. ALDI-TOF mass spectrometric analysis revealed that Alab™ was *Lactobacillus helveticus* with scores of 2.595 (score ≥ 2.0 = identification to the species level [37].
BLAST search confirmed that the 16S rRNA gene sequences of *Lactobacillus helveticus* (1,406 bp; GenBank accession nos. ON834457) shared 98.7% similarity to *Lactobacillus helveticus*2595 (NBRC 15019) isolated in China. (Fig 1).



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BLASTN 2.13.0+

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000),
"A greedy algorithm for aligning DNA sequences", J Comput Biol 2000;
7(1-2):203-14.


Database: 16S ribosomal RNA (Bacteria and Archaea type strains)
          26,881 sequences; 38,967,640 total letters


Query= KJM_HE

Length=1436
                                                                    Score     E
Sequences producing significant alignments:                        (Bits)  Value

NR_042111.1 Lactobacillus gallinarum strain ATCC 33199 16S riboso...  2606    0.0
NR_117061.1 Lactobacillus gallinarum strain ATCC 33199 16S riboso...  2606    0.0
NR_113261.1 Lactobacillus gallinarum strain JCM 2011 16S ribosoma...  2606    0.0
NR_113719.1 Lactobacillus helveticus strain NBRC 15019 16S riboso...  2595    0.0
NR_042439.1 Lactobacillus helveticus DSM 20075 = CGMCC 1.1877 16S...  2595    0.0
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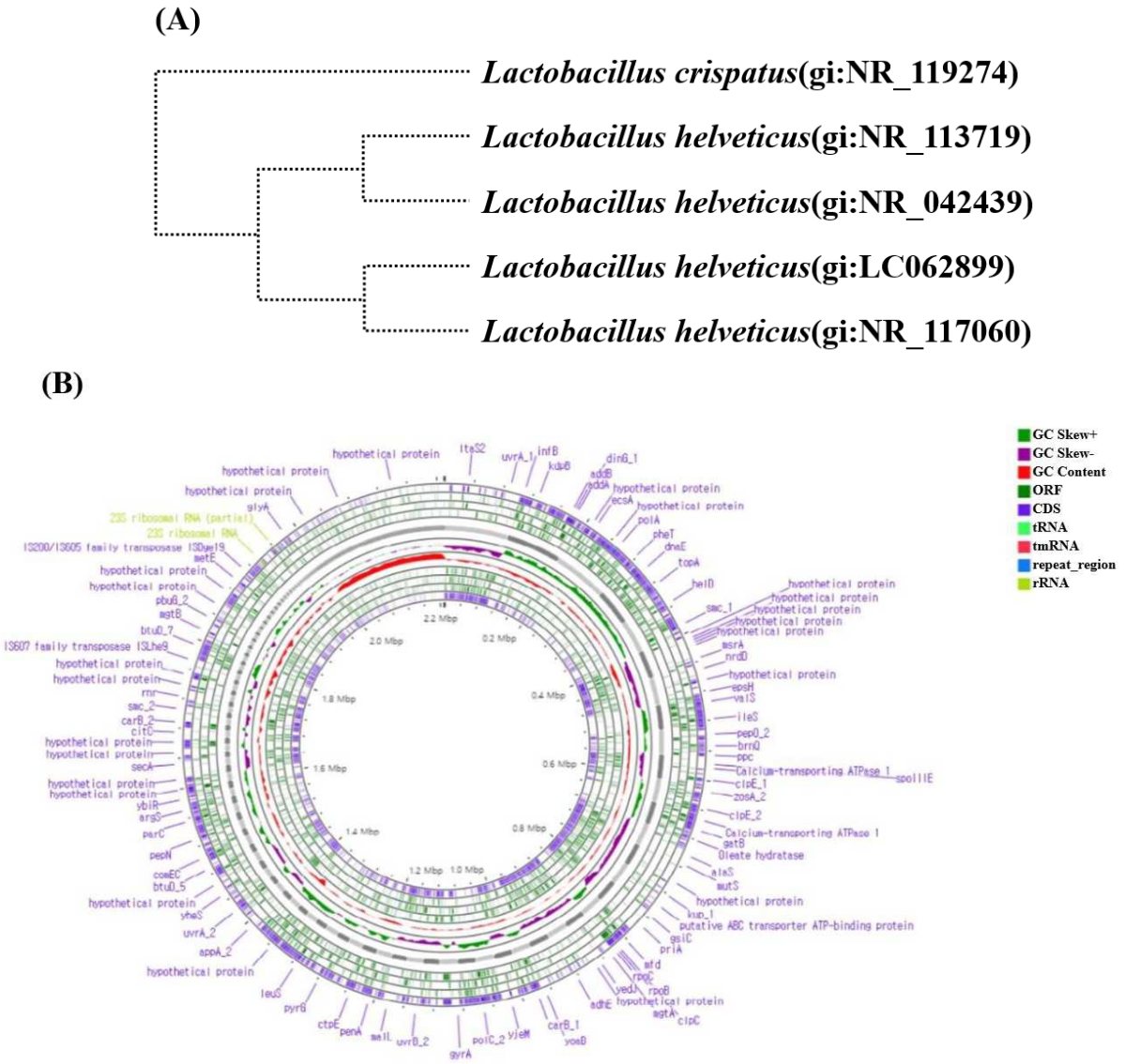


Fig 1: Phylogenetic tree of, **Alab^{TM} based on 16S rRNA (A)/DNA full genome (B)sequences constructed using the neighbor joining method.** The numbers above the branches are bootstrap values. Bars indicate the number of nucleotide substitutions per site. Isolates used in this study are indicated in bold and with arrows.

3.2. Survival of Alab^{TM} at weak acidic pH

In most humans, the digestive juices in the midgut generally fall within the pH range of 4.5-5.5 [38–39]. To evaluate the survival of Alab^{TM} under weak acidic conditions, we assessed the survival of Alab^{TM} in MRS broth at pH 5.0 and 5.5 for 24 hrs. The viable cell counts of the Alab^{TM} at pH 4.5, used as a control, did not change over 24 h, and were similar even at pH and 5.5 (Fig 2). This result indicate that tested strain is resistant to the simulated human gut pH environment and that species specificity.

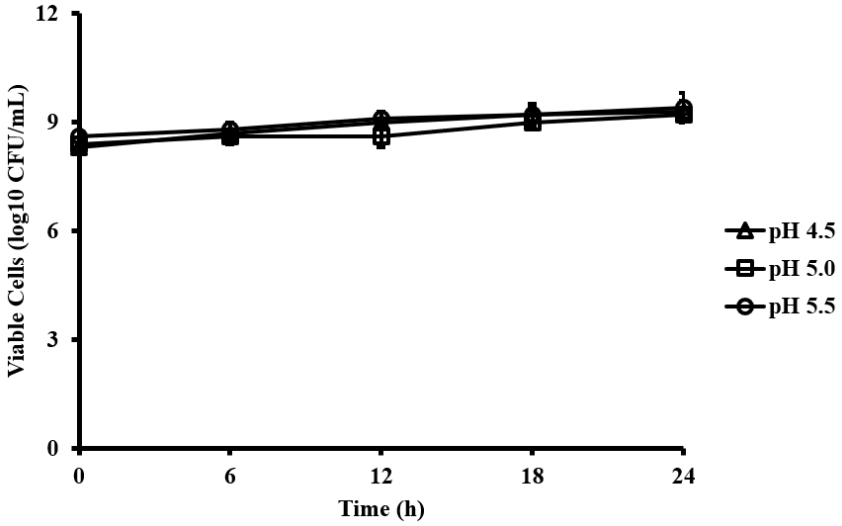


Fig 2: Survival of Alab™ at weak acidic pH. Viable cells of Alab™ over time in MRS broth at pH 4.5, pH 5.0, or pH 5.5. Values are means of data from triplicate experiments each of which contained three technical replicates, with the standard deviation (SD) indicated by vertical bars.

3.3. Survival of Alab™ in simulated gastric juice and bile salt solution of humans

We assessed the viability of Alab™ in a low pH environment simulating the human gut to determine critical probiotic availability. To evaluate the survival of Alab™ under acidic conditions, we assessed the survival of Alab™ in simulated gastric juice (pH 2.2 or 2.5) containing 1% pepsin for 120 min. LAB had high survival rates at pH 2.5 over 120 min of exposure (Fig 3). This result indicates that tested Alab™ exhibit resistance to the gastric juice environment. To evaluate whether bile salts reduce the survival of Alab™, the Alab™ was assessed for survival in MRS media containing 0.1% and 0.5% bile salt (oxgall). A tested Alab™ isolates showed high survival rates after 24 hrs of exposure to 0.1% and 0.5% oxgall (Fig 4A and 4B). This result indicate that the Alab™ have significant tolerance to bile salts.

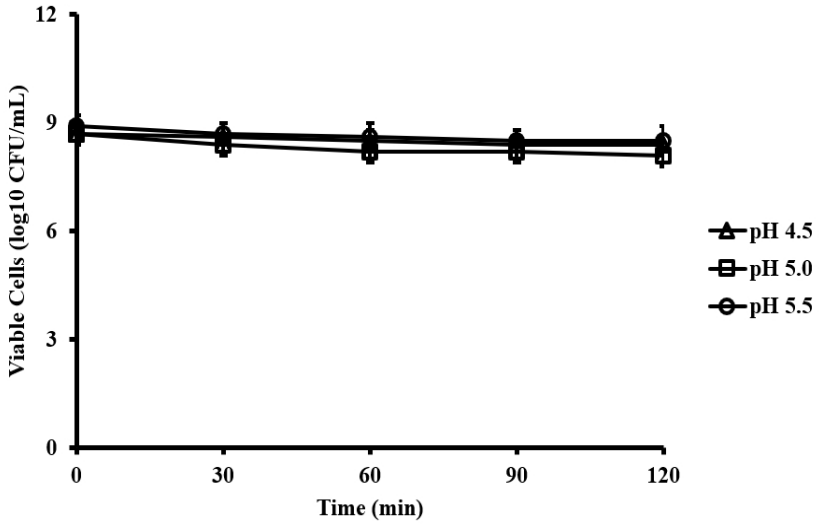


Fig 3: Survival of Alab™ in simulated gastric juice of humans. Viable cells of Alab™ over time in MRS broth containing 1% pepsin at pH 2.5. Values are means of data from triplicate experiments, with the standard deviation (SD) indicated by vertical bars.

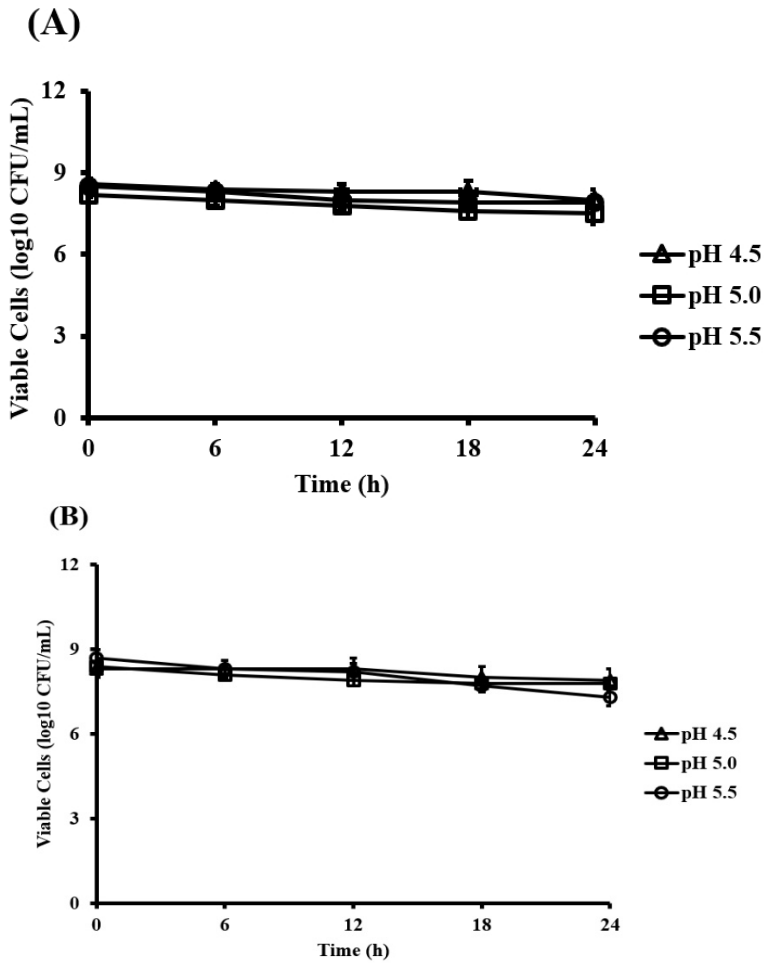


Fig 4: Survival of Alab™ in simulated bile juice of humans. Viable cells of Alab™ over time in MRS broth containing 0.1% (w/v) (A) or 0.5% (w/v) (B) bile salt. Values are means of data from triplicate experiments, with the SD indicated by vertical bars.

3.4. Effects of Alab™ on Locomotor Activity

Actograms were used to visualize the effects of Alab™ on locomotor activity (Fig. 5A). For Alab™, locomotor activity decreased during all phases in a dose dependent manner, and the activity in the 20 µg/mL Alab™ group was greatly reduced, showing a calm state with the decreased black areas; the activities during both night and daytime were significantly decreased by 54 and 78%, respectively, by 20 µg/mL Alab™ treatment (p<0.05) (Fig. 5B, 5C). In addition, lower doses of Alab™ (5, 10 µg/mL) produced a significant decrease in locomotor activity compared to the control group during daytime. This result indicates that Alab™ effectively displayed a sedative function that favored sleep promotion.

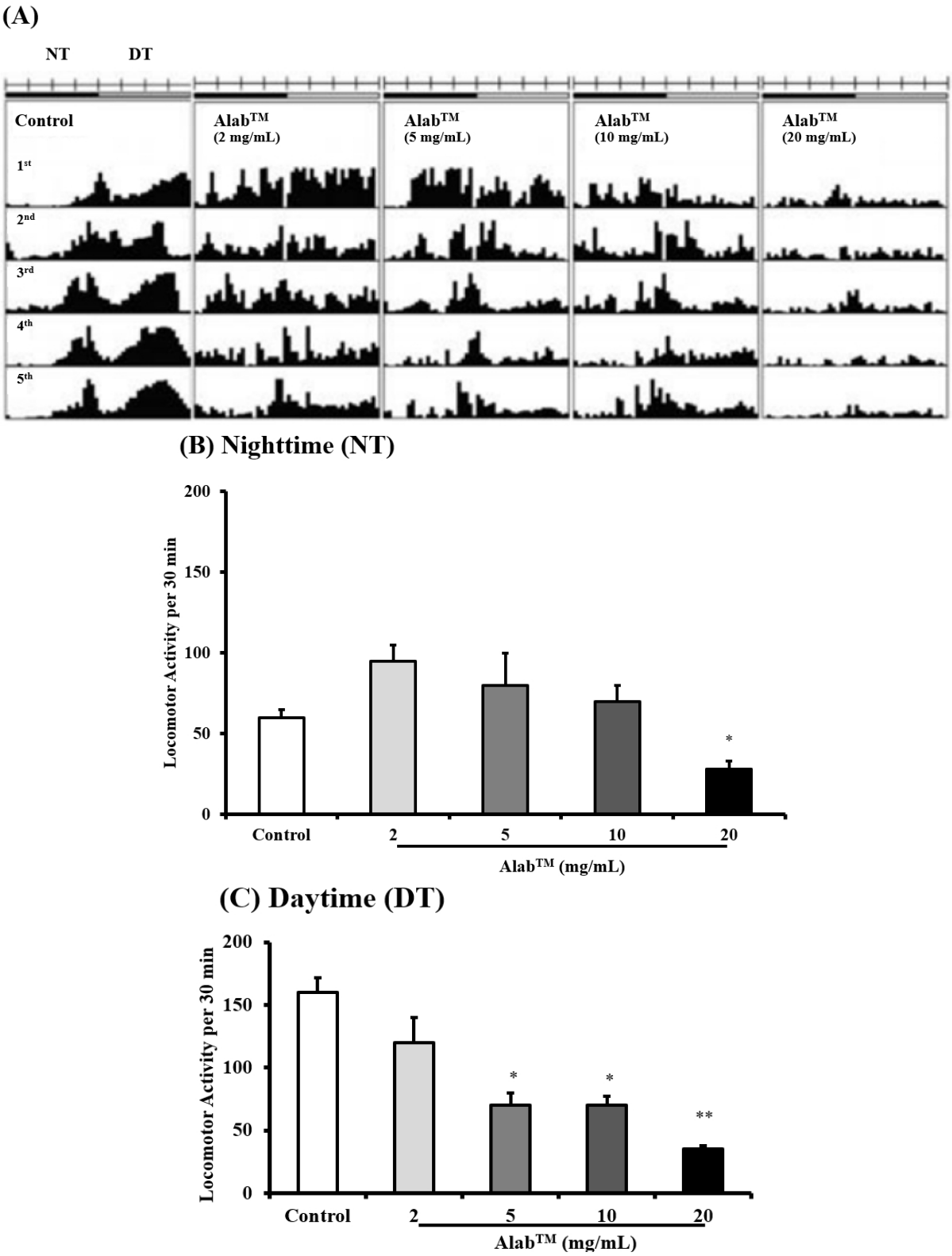


Fig 5: Effects of Alab™ on Locomotor Activity in Fruit Flies. This experiment was performed under constant darkness (DD) for 5 days (3 d: adaptation with normal diet, 5 d: experiment with treatments of Alab™ in sucrose agar media). (A) Typical actograms of individual control flies (n=20) and flies exposed to Alab™ (n=16) by dose. Average activity in a 30 min interval was calculated over 5 d. Black/white bars on top of the actograms indicate dark (22:00 to 10:00) and light (10:00 to 22:00) phases. (B) Activity during dark phases and (C) activity during light phases. Values indicate the means ± S.E.M. for each group. Symbols indicate statistically significant differences versus Control (*p<0.05, **p<0.01).

3.5. Effect of Alab™ on Sleep Behavior

The effects of Alab™ on dark phase activity, number of sleep bouts, and total dark phase sleep were examined (Fig. 6). With the DAM system for the individual sleep behavior, total movement of flies seemed to decrease in the Alab™ treated group, although it was not statistically significant (Fig. 6A). Alab™ treated group showed significantly higher levels of sleep bouts, which are an interruption of sleep, compared to the control (Fig. 6B). The Alab™ showed lower sleep bouts, but still higher than normal control. For total nighttime sleep duration, Alab™ treatment seems to increase sleep time compared to the control, but statistical significance was not observed (Fig. 6C).

Alab™ treatment also did not show the significant difference in total nighttime sleep compared to the control. In the LAM system including social behavioral activity, movement activity in the Alab™ group appeared to decrease, although it was not statistically significant (Fig. 6D). On the other hand, Alab™ treated flies demonstrated a significant increase in total nighttime sleep (Fig. 6E). This result showed that the Alab™ had a sleep promoting effect in social behavioral condition.

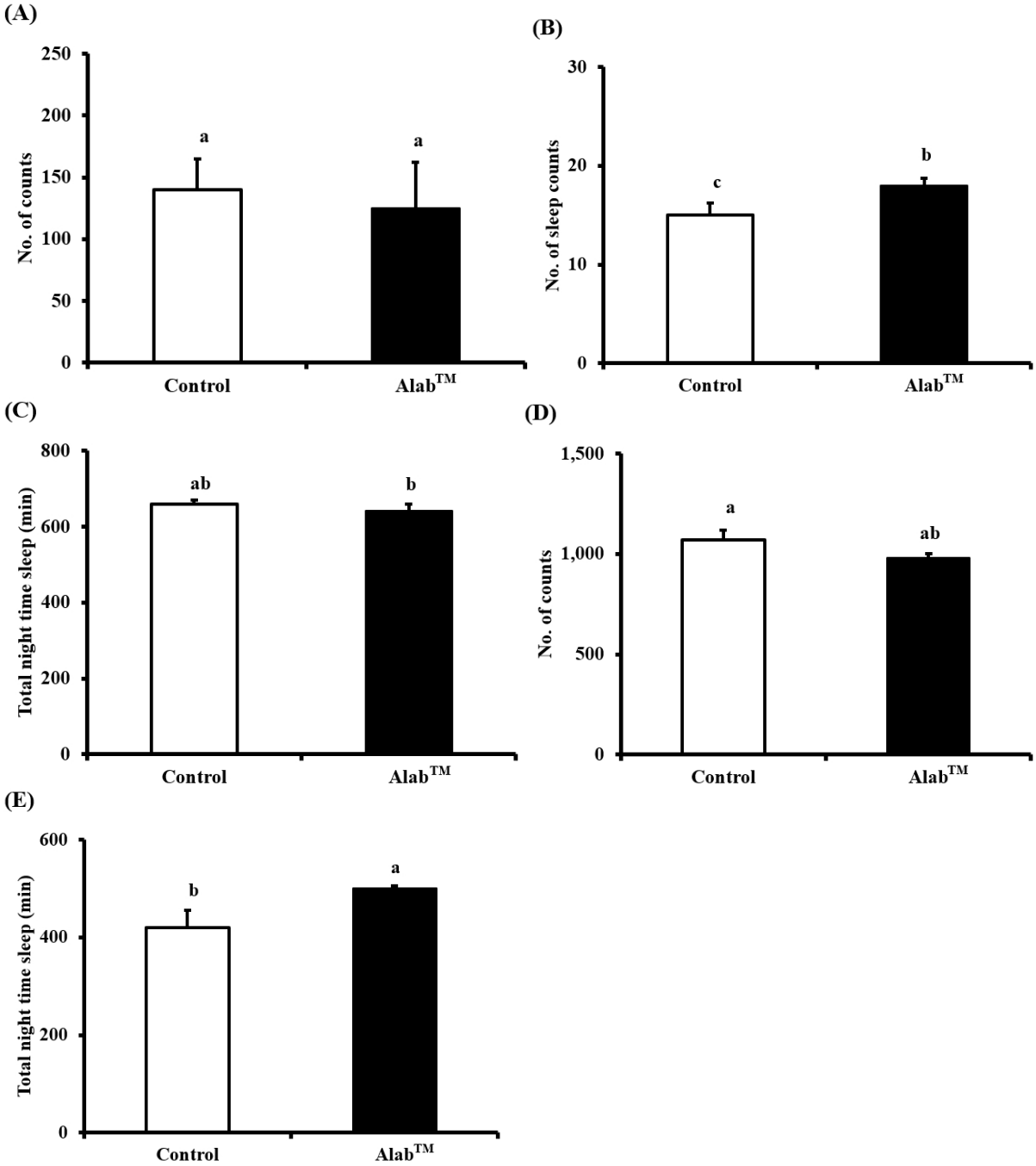
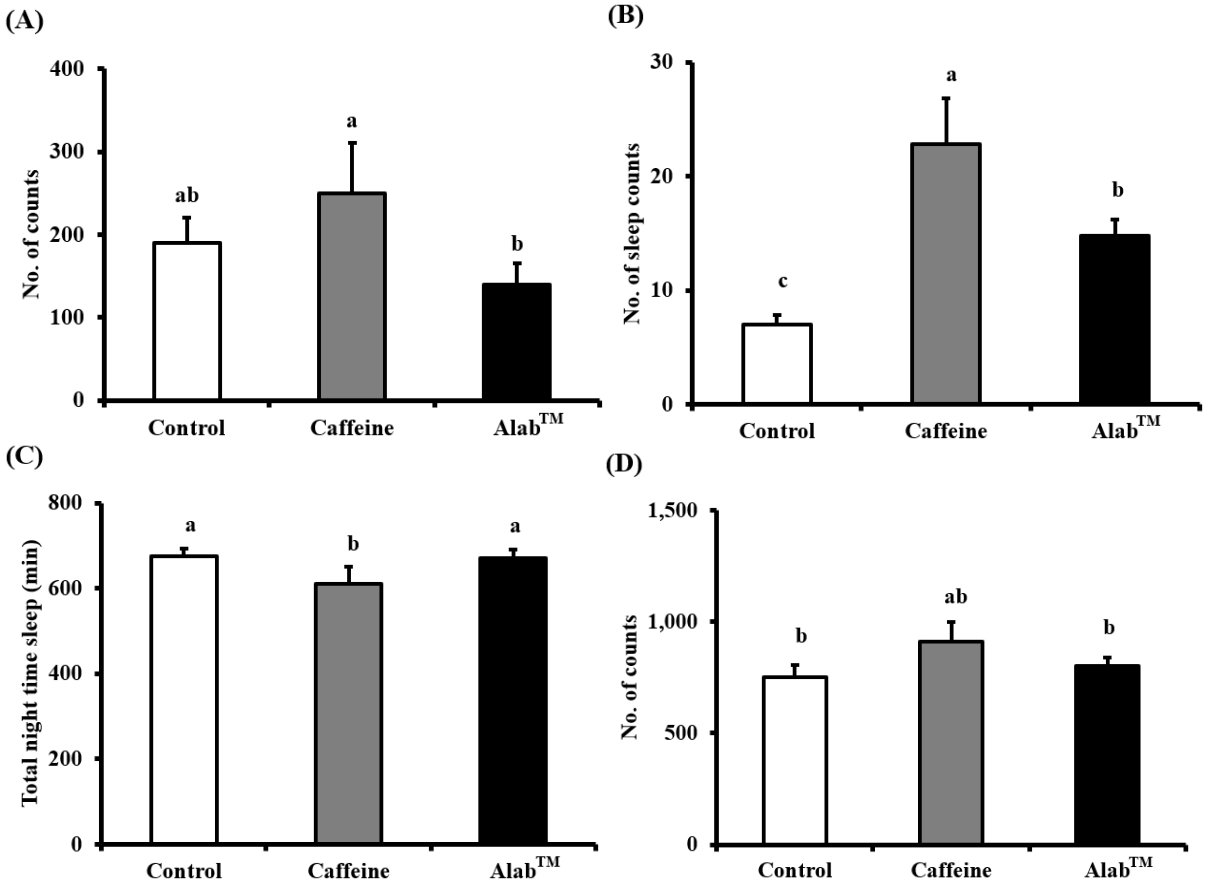


Fig 6: Effects of Alab™ on Sleep Behavior in Fruit Flies.

This experiment was performed under constant darkness (DD) for 8 days (3 d: adaptation, 5 d: experiment). (A) Dark phase activity, (B) number of sleep bouts, and (C) duration of dark phase sleep of the control group (sucrose agar media group), 10 mg/mL Alab™ treatment group using the Drosophila Activity Monitoring (DAM) system. (D) Dark phase activity and (E) amount of dark phase sleep of the control group (sucrose agar media group), 10 mg/mL LAB treatment group using the Locomotor Activity Monitoring (LAM) system. Values represent the means ± S.E.M. for each group. Different letters indicate significant differences.

3.6. Effect of aAlab™ on Sleep Behavior in a Caffeine Induced Awake Model

Dark phase activity and sleep bouts in the caffeine fed group significantly increased compared to the control group while total sleep time showed a significant decrease (Fig. 7A–7C). Administration of the Alab™ showed a marked reduction in total movement activity (Fig. 7A). Number of sleep bouts also significantly decreased with the Alab™ compared to the caffeine treated group (Fig. 7B). Meanwhile, total sleep time of the Alab™ group in the dark phase was significantly increased compared to the caffeine only group (Fig. 7C). In LAM system, total movement of the Alab™ flies was similar level to normal group, which is slightly lower than caffeine only group (Fig. 7D). In addition, dark phase sleep time of the Alab™ group exhibited a significant increase compared with caffeine only exposed group (Fig. 7E). Collectively, this result showed that the Alab™ was effective in sleep promotion in caffeine induced awake model.



(E)

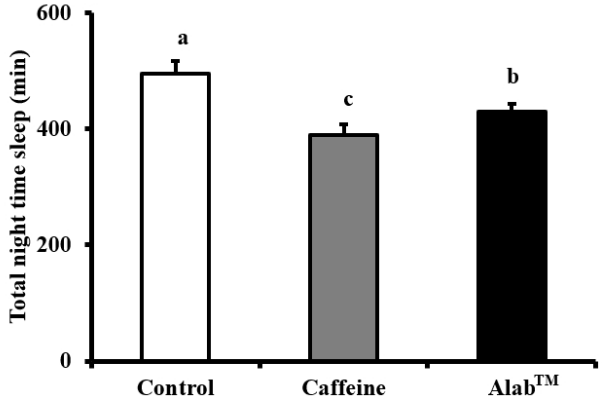


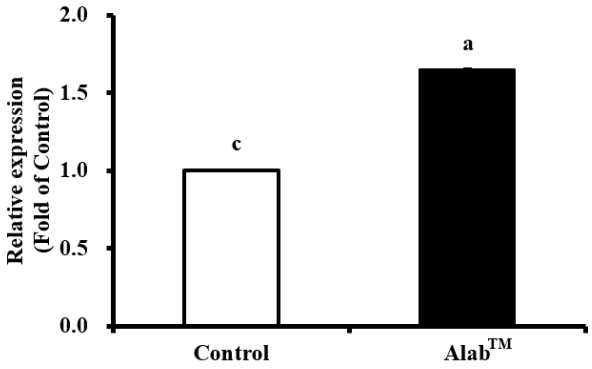
Fig 7: Effect of Alab™ on Caffeine Induced Wakefulness in Fruit Flies.

This experiment was performed under constant darkness (DD) for 4 days (3 d: adaptation, 4 d: experiment). (A) Dark phase activity, (B) number of sleep bouts, and (C) amount of dark phase sleep of the control group (sucrose agar media group) 20 mg/mL Alab™ with the 10 mg/mL caffeine treatment group using the Drosophila Activity Monitoring (DAM) system. (D) Dark phase activity and (E) amount of dark phase sleep of the control group (sucrose agar media group), 20 mg/mL Alab™ with 10 mg/mL caffeine treatment group using the Locomotor Activity Monitor (LAM) system. Values represent the means±S.E.M. for each group. Different letters indicate significant differences.

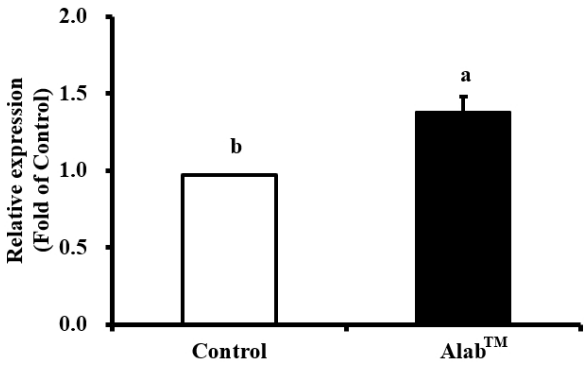
3.7. Effects of Alab™ on mRNA Levels of Neurotransmitter Signaling

Transcript levels of Resistant to dieldrin (Rdl), which is a *Drosophila* GABAA receptor, in Alab™ group was significantly increased compared to the control group (Fig. 8A). The Alab™ increased Rdl mRNA expression by over 50% compared to control group (Fig. 8A). In addition, mRNA levels of GABAB receptor 1 (GABAB- R1) also showed a significant increase in the Alab™ group (Fig. 8B). However, the mRNA levels of these receptors in the treatment group decreased or remained similar to controls. Moreover, for GABAB receptor 2 (GA BA B-R2) and 5-hydroxytryptamine receptor 1A (5-HT1A) mRNA expressions, the Alab™ group expressed a higher level than the control group, but significance was not observed (Fig. 8C, 8D).

(A) Rdl



(B) $GABA_B$ - R1



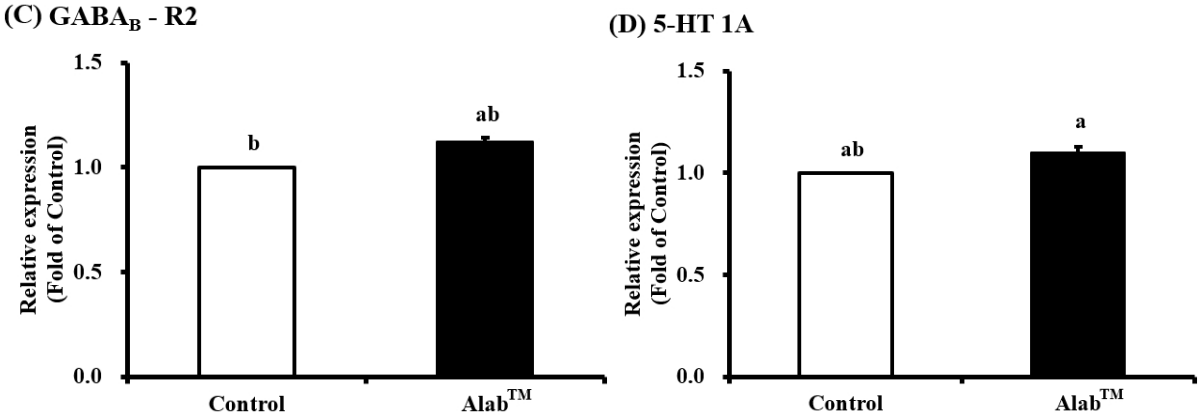


Fig 8: Effect of Alab^{TM} on Rdl, GABAB-R1, GA BA B-R2, and 5-HT1A mRNA Expression in Fruit Fly Heads. Experiments were performed after exposure to a 12 : 12 hrs light : dark cycle for 2 weeks. Rdl; Resistant to Dieldrin, GABAB-R1; GA BA B receptor 1 GABAB-R2; GABAB receptor 2,5-hydroxytryptamine A receptor 1 (5-HT1A). Control; sucrose agar media group, Alab^{TM}; Valerian 20 mg/mL in sucrose agar media. Values represent the means ± S.E.M. from 150 flies per group. Different letters indicate significant differences.

IV. DISCUSSION

Lactobacillus helveticus is mainly isolated from plants and the environment. Thisis used for cheese production and fermentation of foods including vegetables, meat, and wine [37]. In addition, this bacterium has been genetically modified for the treatment of human diseases [38]. *L. helveticus* has GRAS status and is beneficial to humans [37]. *L. helveticus* was identified in *Aloe vera* (*Aloe Barbadensis* Miller) by 16S amplicon sequencing [39]. The *L. helveticus* is isolated mainly from the guts of healthy humans and many animals.

We focused on whether Alab^{TM} isolated from the Jeju *Aloe vera* can function as LAB. LAB benefit host cells only if they survive low pH conditions (pH 2.0–2.5) and toxic bile salts during passage through the gastrointestinal tract and adhere well to human cells [40, 41]. The Alab^{TM} investigated in this study survived at densities of > 4.2 log10 CFU/mL in simulated gastric juice at pH 2.2 (Fig 3). Alab^{TM} belong to the same species of *L. helveticus*, but showed different sensitivities to low pH. This result was consistent with previous research, which indicates that strains of the same *Lactobacillus* species may exhibit differences in viability at low pH and strain dependent survival [40, 42]. Alab^{TM} survived well in simulated gastric juice at pH 2.5 or 0.5% bile salt solution (Fig. 3 and 4). Therefore, we suggest that this Alab^{TM} obtained from the leaves of the Jeju *Aloe vera* seedlings exhibit the critical characteristics of probiotics. According to the viability assay in the simulated human gut pH, i.e., weakly alkaline environment, Alab^{TM} survived well in MRS broth at pH 8 (Fig 2).This result indicate that bacteria fed to human proliferated and successfully colonized the gut. In this study, we showed the total colony population of Alab^{TM} instead of LAB localization in the gut of human, as it is currently unknown whether LAB are restricted to specific gut sections. Interestingly, these follow-up studies on this issue will have implications for understanding the natural ecology of Alab^{TM} and will provide new opportunities for industrial strain development. In this study, we isolated and identified Alab^{TM} from the Jeju *Aloe vera* in Korea, and demonstrated that this bacterium has the essential features of LAB. The present study is the first to report Alab^{TM} isolated from the Jeju *Aloe vera* with potential application as probiotics.

Insomnia is prevalent among people; over 30% of the world's population have symptoms of insomnia, and 10% of the people are known to have severe chronic insomnia [43]. Many studies have shown that insomnia causes not only risks of mental or physical health [44] but also poor quality of life, increased occupational errors, and increased industrial accidents [44]. Although pharmaceutical treatments have been suggested, the demand on natural product targeting study has been increased owing to drug mediated side effects.

This study showed the potential of a Alab^{TM} as a sleep promoting agent in a *Drosophila* model. *L. helveticus* is recognized for its sedative and soothing medicinal properties and is used to ease symptoms of insomnia. Recent studies showed that *L. helveticus* influences sleep through various factors in rats and humans [45, 46], but its efficacy on sleep has not been demonstrated in a *Drosophila* model. *L. helveticus* has also been used as a dietary supplement for mood and sleep disturbances [45, 46]. Several studies reported the sedative effects of *L. helveticus* [46], but a systematic assessment on sleep has rarely been done. In current study, sedative effect of Alab^{TM} on the locomotor activity was identified (Fig. 5), and sleep enhancing effect of Alab^{TM} was observed as a significant increase compared to the normal control in DAM and LAM system, respectively (Fig. 6C, 6E). The Alab^{TM} was shown to have a significant sleep promoting effect in normal condition of *Drosophila* in LAM system (Fig. 6E). This result showed that the Alab^{TM} has combinational or synergistic effect on the sleep promotion, and previous reported sedative effects of *L. helveticus*[27, 28] can be connected to the sleep promoting effect by Alab^{TM}.

Caffeine is the most widely used material to induce physical wakefulness. Caffeine functions as an antagonist for the adenosine 2A subunit [47]. Moreover, caffeine affects arousal by modulating protein kinase A (PKA) and cAMP in flies [48]. Thus, caffeine is an appropriate method to enhance arousal in a *Drosophila* model. The Alab™ treatment exhibited less movement activity and longer sleep time than caffeine only treated group in the caffeine induced awake condition (Fig. 7A, 7C). Unlike normal condition, sleep promoting effect of the Alab™ was observed in both DAM and LAM systems (Fig. 7C, 7E). Thus, this result indicated that Alab™ can play more important role in sleep promotion on caffeine induced awake conditions than normal condition.

A recent study showed the possibility of Alab™ in the treatment of insomnia patients [49], supporting our data. In particular, it has been known to bind directly to GABA A receptors that favor sleep [50]. GABA plays an important role in many rhythmic activities by modulating arousal and relaxation. Our data showed that a Alab™ up regulates both the GABA receptor and serotonin receptor (Fig. 8). Particularly, this mixture was shown to regulate GABA receptors more strongly than serotonin receptors (Fig. 8).

Current study describes the effect of the Alab™ on the locomotor activity and sleep durations to determine sleep promoting capacity of the Alab™. However, its effect on sleep patterns including rapid eye movement (REM) and non-rapid eye movement (NREM), and the effect on sleep induction were not handled in this study. Sine these sleep related data would tell more details including effects of the Alab™ on the quality of sleep, studies using mammalian models would be performed in the next.

Our results demonstrated that a Alab™ promotes sleeping with the reduction of dark phase activity compared to the control condition or single administration of the Alab™ in fruit fly model. This sleep promoting effect of the Alab™ occurred via regulation of neuromodulator signaling components such as Rdl, GABAB, and 5-HT1A receptors.

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