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EFFECTS OF A MIXED MICROBIAL CULTURE OF *LACTOBACILLUSPARACASEI, PICHIAMEMBRANIFACIENS,* AND *SACCHAROMYCESCEREVISIAE* ON AUTONOMIC NERVE ACTIVITIES

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ABSTRACT

Certain fermented foods have been shown to have positive effects on health. To determine the effects of a mixed microbial culture of *Lactobacillus paracasei*, *Pichia membranifaciens*, and *Saccharomyces cerevisiae* (LBS culture), we focused on the effect of dermal application of LBS culture on adrenal sympathetic nerve activity (ASNA) and cutaneous arterial sympathetic nerve activity (CASNA),and the effect of intra gastric administration of LBS culture on celiac vagal nerve activity (CVNA) in urethane-anesthetized rats. Results revealed that 1) Dermal application of 100-fold diluted LBS culture significantly reduced both ASNA and CASNA, and 2) intra gastric administration of 10,000-fold diluted LBS culture significantly elevated CVNA. Since the suppression of ASNA leads to decrease in adrenaline and noradrenaline secretions, and since these hormones have been reported to elevate blood glucose and blood pressure, our findings suggest that LBS culture may lower blood glucose and blood pressure. Suppression of CASNA causes dilatation of the cutaneous arteries and increases water retention. Therefore, the results of this study suggest that LBS culture may increase water retention in the skin. Moreover, since an increase in CVNA suggests an improvement in gastrointestinal peristalsis, the present observations suggest that LBS culture may have a laxative effect.

Keywords: *Lactobacillus paracasei; Saccharomyces cerevisiae*; *Pichia membranifaciens*; adrenal sympathetic nerve activity; cutaneous arterial sympathetic nerve activity; celiac vagal nerve activity

1. INTRODUCTION

Autonomic nerves regulate various bodily functions. Previous reports suggest that several kinds of *Lactobacilli* affect physiological functions, including the blood glucose level, blood pressure, appetite, lipolysis, body temperature, natural killer cell activity, gastrointestinal transit, and cutaneous water retention, via changes in the autonomic nerve activity, which is involved in regulating these respective functions [1-9].

*Lactobacillus paracasei*is a gram-positive, non-spore forming, homo fermentative bacterium, and a common inhabitant of the human intestinal tract [10,11]. *Saccharomyces cerevisiae*, commonly known as baker's yeast, is a single-celled eukaryote. *Pichia membranifaciens*, a budding yeast, was isolated from spoiled wines and a cellar environment in Patagonia [12]. *Lactobacillli* commonly excrete organic acids, and acids lower the pH, which promotes yeast growth[13].Therefore, *Lactobacillli* and yeasts may synergistically promote the growth of each other. Supporting this association, multi-strain and/or multi-species probiotics have been shown to be more effective than monostrain probiotics [14].In this regard, LBS culture, aheat-inactivated microbial culture comprising *L. paracasei*, *P. membranifaciens*, and *S.cerevisiae*, was shown to promoteimmune responses in carp [15].In order to determine the effectsof LBS culture (a mixed microbial culture of *L. paracasei*, *P. membranifaciens*, and *S.cerevisiae* (Litanial Bio Science, Co., Ltd. Hyogo, Japan)),we studied the effects of dermal application of an LBS culture on the efferent activities of the adrenal sympathetic nerve and cutaneous arterial sympathetic nerve in urethane-anesthetized rats. Furthermore, the effect of intra gastric administration of LBS culture on the efferent activity of the celiac vagal nerve, which innervates the small intestine, was also examined. The results are described in this report.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar rats, weighing 250–300 g, were used for the experiments. The animals were housed in a room maintained at 24°C ± 1°C and illuminated daily for 12 h (07:00–19:00). Food and water were available *ad libitum*. Rats were allowed to acclimatize to the environment for at least 1 week prior to the experiments. All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of ANBAS Corporation.

2.2 Preparation of LBS culture and control solution

The LBS culture was purchased from Litanial Bio Science, Co. Ltd. (Hyogo, Japan)[7]. This culture was prepared by co-cultivation of *L. paracasei*, *P. membranifaciens*, and *S.cerevisiae*in a rice grain broth supplemented with 5% dextrose at 30°C for 24 h. The presence of each microbial

strain was confirmed by biochemical tests and PC Ramplification of 16S, 26S, and 18S ribosomal RNA fragments, respectively. This culture contained 5.4 x 10^7 cells/ml of *L. paracasei*, 2.4 x 10^6cells/ml of *P. membranifaciens*, and 4.4 x 10^7cells/ml of *S.cerevisiae*. The number of bacterial and yeast cells in the mixed microbial culture was microscopically determined using a hemocytometer. The culture was inactivated by heating at 121°C for 15 min. Sterility was assessed by cultivation of the microbial mixture on heart infusion (HI) agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan).

2.3 Experimental procedure

The general procedures have been described previously [16]. The animals were anesthetized with an intra peritoneal (IP) injection of 1 g/kg urethane and were cannulated intra tracheally. The animals were fixed in a stereo taxic apparatus, where body temperature was maintained at 37°C to 37.5°C using a heating pad.

After laparotomy, the left adrenal sympathetic nerve was ligated at its distal end and connected to a pair of silver electrodes as described previously [17].After longitudinal incision of the left femoral skin, the cutaneous arterial sympathetic nerve was ligated at its distal end and connected to a pair of silver electrodes as described previously [2].The efferent celiac vagal nerve that innervates the small intestine was identified and exposed after laparotomy [3]. The distal end of the celiac vagal nerve was ligated and connected to a pair of silver electrodes. The recording electrodes were immersed in liquid paraffin oil to prevent dehydration and for electrical insulation. The animals were allowed to stabilize for 30 min to 60 min after placing the recording electrodes. Then, LPS was applied through the skin or administered intra gastrically. Electrical changes in adrenal sympathetic nerve activity (ASNA), cutaneous arterial sympathetic nerve activity (CASNA), and celiac vagal nerve activity (CVNA)were amplified and monitored by an oscilloscope. The raw data from nerve activity were converted to standardized pulses by a window discriminator that eliminated background electrical noise. The data were converted from analog to digital format and then stored on a PC as described previously [16].

Once an animal had stabilized, baseline (0 min) ASNA, CASNA, and CVNA measurements were obtained 5 min prior to the beginning of dermal application or intragastric administration of LBS culture or liquid broth.

For the dermal application, about 200 mg of cotton soaked in 2 ml of LBS culture (original concentration or diluted in liquid broth) was applied to the exposed right femoral skin, tail skin, and plantar skin at a ratio of 0.8:0.8:0.4:0.4, with 0.8 ml (80mg of cotton; right femoral skin) to 0.8 ml (80mg of cotton; tail skin) to 0.2 ml (20mg of cotton; right plantar skin) to 0.2 ml (20mg of cotton; left plantar skin), and the cotton was covered by polyvinylidene chloride film. For the

intragastric administration, 1 ml/300 g body weight of LBS culture (original concentration or diluted in liquid broth) was injected into the stomach using a stomach cannula made of polyethylene tubing (PE60, Becton Dickinson & Co.). Liquid broth, the LBS culture medium, was used as a control for dermal application and intragastric administration of the culture.

Autonomic nerve activities (ASNA, CASNA, and CVNA)were measured at 5-min intervals over a 60-min test period, from the beginning of the dermal application to intragastric administration.

2.4 Statistical analysis

Data were expressed as percentages of the baseline (0 min) values, because of inter-individual variability in pre-injection states, and their mean ± standard error of the mean (S.E.M.) were calculated. The Mann-Whitney U test was used to compare absolute baseline values (spikes/5 s) in each group. Analysis of variance (ANOVA) with repeated measures was used to determine the statistical significance of differences among the ASNA, CASNA, and CVNA values from 5-60 min as groups with $P< 0.05$ considered statistically significant.

3. RESULTS

3.1 Effect of LBS culture application to the skin on ASNA

In a preliminary study, effects of dermal applications of 2 ml each of 5 different concentrations of LBS culture, the original concentration, and 10-, 100-, 1000-, and 10000-fold dilution sin liquid broth, on ASNA were examined in urethane-anesthetized rats (Fig. 1A). As seen in Fig. 1A, dermal application of 100-fold diluted LBS culture showed the strongest suppression of ASNA among the 5 concentrations of LBS culture, and the lowest ASNA value (74.6 %) were observed at 55 min at this dilution (Fig. 1A).

Therefore, the effect of applying 2 ml of 100-fold diluted LBS culture to skin on ASNA was examined. Fig. 1B shows representative ASNA recordings, and Fig. 1C shows percent changes in ASNA after dermal application of liquid broth (control) or 100-fold diluted LBS culture. The application of liquid broth did not change ASNA significantly. The highest ASNA value (102%±7%) was observed 25 min after dermal application began, and the lowest ASNA value (94%±6%) was observed at 45 min after application. ASNA values during the test period stayed between these 2 values (Fig. 1C). In contrast, applying 100-fold diluted LBS culture to skin gradually lowered ASNA, which decreased to its lowest value (72.9% ± 1.0%) 55 min after application began (Fig. 1C). A significant difference ($P<0.0005$, $F=59.5$) was detected by ANOVA with repeated measures between ASNA values following application of liquid broth or 100-fold diluted LBS culture. The baseline (0 min) values of ASNA in these 2 groups were 226±21 spikes/5 s (control ;n = 3) and 216±18 spikes/5 s (100-fold dilution; n = 3). The

difference between these baseline values was not statistically significant by the Mann-Whitney U test.

3.2 Effect of LBS cultureapplication to the skin on CASNA

Fig. 2A shows changes in CASNA after skin application of 2 ml each of 5 different concentrations of LBS culture, the original concentration, and 10-, 100-, 1000-, and 10000-fold dilution sin liquid broth, in apreliminary study. As seen in Fig. 2A, among the 5 rats used in this study, dermal application of 100-fold diluted LBS culture showed the strongest suppression of CASNA with the lowest CASNA value (74.3%) observed at 60 min after LBS application.

Therefore, the effect of applying 2 ml of 100-fold diluted LBS culture to skin on CASNA was examined. Fig. 2B shows representative CASNA recordings, and Fig. 2C shows the percent changes in CASNA after dermal application of liquid broth (control) or 100-fold diluted LBS culture. The application of liquid broth did not change CASNA significantly. The lowest CASNA value (97%±3%) was observed 10 min after the application began, and the highest CASNA value (106%±7%) was observed at 55 min. CASNA values during the test period stayed between these 2 values (Fig. 2C). In contrast, applying100-fold diluted LBS culture to skin gradually lowered CASNA, which decreased to its lowest value (90% ± 8%) (Fig. 2C) 60 min after the application began. A significant difference ($P<0.0005$, $F=38.0$) was detected by ANOVA with repeated measures between CASNA values following application of liquid broth or 100-fold dilutedLBS culture. The baseline (0 min) values of CASNA in these 2 groups were 250±14 spikes/5 s (control;n = 3) and 250±37 spikes/5 s (100-fold dilution;n = 3).The difference between these baseline values was not statistically significant by the Mann-Whitney U test.

3.3 Effect of intragastric administration of LBS culture on CVNA

Fig. 3Ashows changes in CVNA after intragastric administration of 1 ml/300 g of body weight of the LBS culture at 5 different concentrations, the original concentration, and10-, 100-, 1000-, and 10000-fold dilution in liquid broth, in a preliminary study. As seen in Fig. 3A, intragastric administration of 1 ml/300g of body weight of 10,000-fold diluted LBS culture showed the greatest elevation in CVNA among the 5 concentrations of LBS culture, with the highest CVNA value (115%) measured at25 min after the beginning of intragastric administration (Fig. 3A).

Therefore, the effect of intragastric administration of 10,000-fold diluted LBS culture on CVNA was examined. Fig. 3B shows representative recordings, while Fig. 3C shows percent changes in CVNA after the administration of 1 ml/300g of body weight of 10,000-folddiluted LBS culture or liquid broth. Intragastric administration of liquid broth did not change CVNA significantly. The highest CVNA value (104%±5%) was observed 5 min after administration began, and the lowest CVNA value (90%±5%) was observed at 55 min. CVNA values during the test period

stayed between these 2 values (Fig. 3C). In contrast, intragastric administration of 10,000-fold diluted LBS culture gradually elevated CVNA, and the CVNA value 45 min after the administration began increased to its highest value (116%±7%)(Fig. 3C). A significant difference ($P<0.0005$, $F=57.6$) was detected by ANOVA with repeated measures between CVNA values 5 to 60 min after administration of liquid broth and 10,000-fold diluted LBS culture. The baseline (0 min) CVNA values in these 2 groups were 256±3 spikes/5 s (control; n=3) and 242±24 spikes/5 s (10,000-fold dilution; n=3). The difference between these baseline values is not statistically significant by Mann-Whitney U test.

4. DISCUSSION

Fermented foods have been known to improve intestinal and immune function, decreasing allergic responses and reducing the risk of colon cancer [18]. Some fermented foods are teeming with beneficial bacteria that boost digestive health. The use of multispecies preparations, containing multiple strains from more than one genus, may be even be more effective than using multi-strain pro biotics [19].

In this study, we found that ASNA and CASNA were suppressed by dermal application of LBS culture in rats (Figs. 1 and 2). Since the suppression of ASNA reduces the secretions of adrenaline and noradrenaline from the adrenal medulla, and because these hormones elevate blood glucose and blood pressure [20], results of this study suggest that LBS culture might reduce blood glucose and blood pressure when applied to the skin. The suppression of CASNA has been suggested to dilate cutaneous arteries, causing an increase in blood flow and water retention in the skin [2,21]. This suggests that LBS culture might increase either cutaneous blood flow or water retention when applied dermally. Moreover, application of LBS culture to skin might be good for wound healing, also, because a rise in cutaneous blood flow increases the supply of oxygen and nutrients to the skin.

Furthermore, intragastric administration of LBS culture was observed to significantly increase the activity of the celiac vagal nerve that innervates the intestine (Fig. 3). Since an increase in CVNA stimulates gastrointestinal transit [3], LBS culture may have a stimulatory effect on gastrointestinal transit and thus be good for constipation.

Recently, excitation of the sympathetic nervous system in pain pathways has drawn attention [22], and the involvement of the sympathetic nervous system in pain sensations has been confirmed in a rat neuropathic pain model [23] and in humans, post-surgically [24]. Furthermore, hyperactivity of the sympathetic nervous system resulting in pain due to erythromelalgia [25] and fibromyalgia [26] in humans has also been suggested. Therefore, it is possible that LBS

culture might alleviate pain by suppressing ASNA. These possibilities should be examined in future studies.

We previously observed that a decrease in CASNA and an increase in cutaneous blood flow due to dermal application of urea were abolished by thioperamide, a histamine H3-receptor antagonist [21]. Therefore, the histamine H3-receptor might be involved in CASNA suppression due to dermal application of LBS culture. Moreover, granisetron, a serotonin 3-receptor antagonist, was found to eliminate an increase in CVNA and stimulation of gastrointestinal transit due to intestinal administration of *Lactobacillus brevis* SBC8803 [3]. Thus, serotonin 3-receptor might be involved in increasing CVNA due to intragastric administration of LBS culture. These possibilities should also be examined in the future.

5. CONCLUSION

We found that dermal application of LBS culture decreased ASNA and CASNA, while intra gastric administration of LBS culture increased CVNA in rats. In light of the present results, LBS culture has the possibility to improve a wide range of skin disorders by dermal application, and to improve peristalsis and ameliorate diabetes, hypertension, and pain sensation. These hypotheses need to be examined further, and the detailed mechanism underlying the effects of LBS culture on ASNA, CASNA, and CVNA should be examined further.

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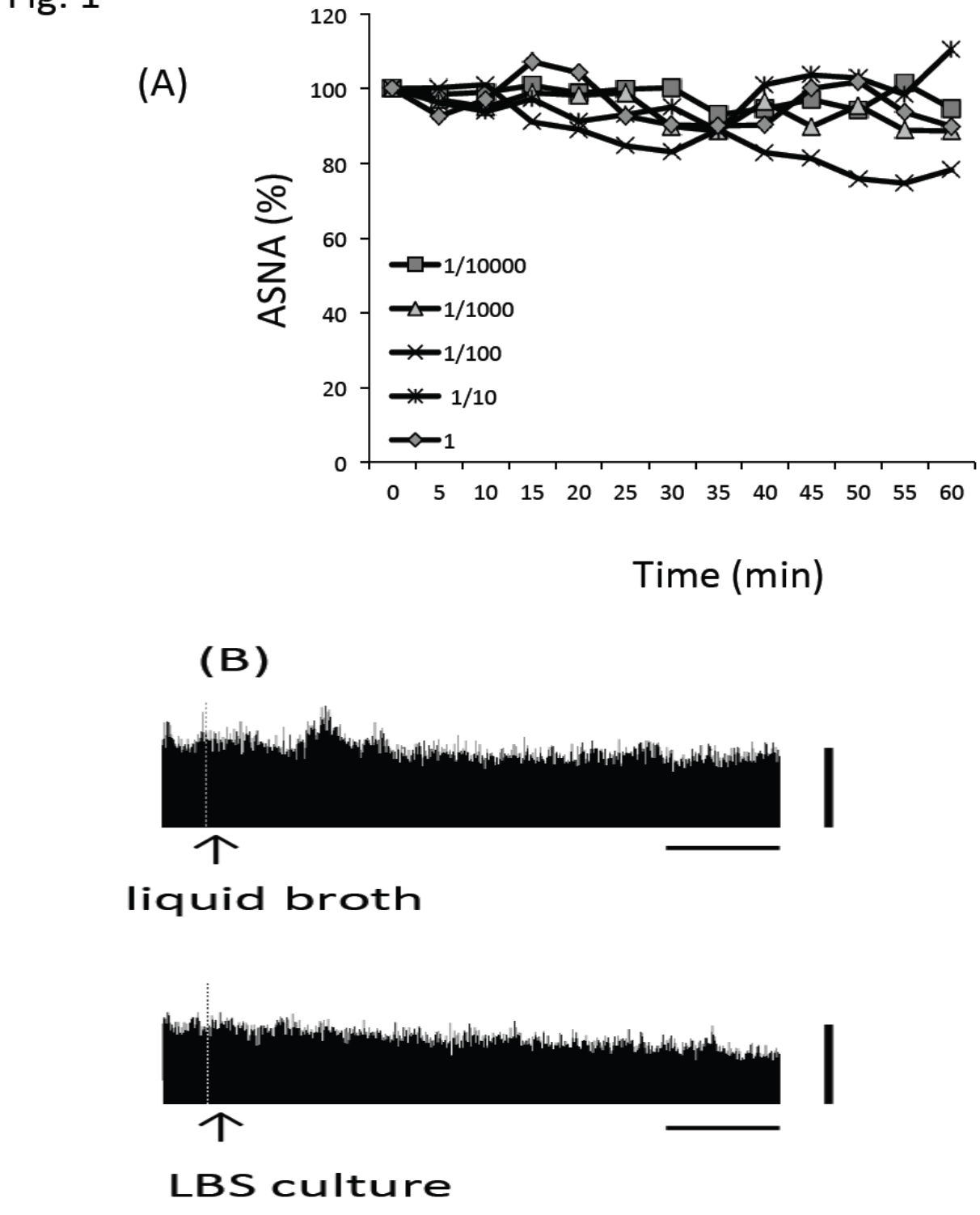
Fig. 1


(A)
ASNA (%)
120
100
80
60
40
20
0
1/10000
1/1000
1/100
1/10
1
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (min)
(B)
liquid broth
LBS culture

(C)

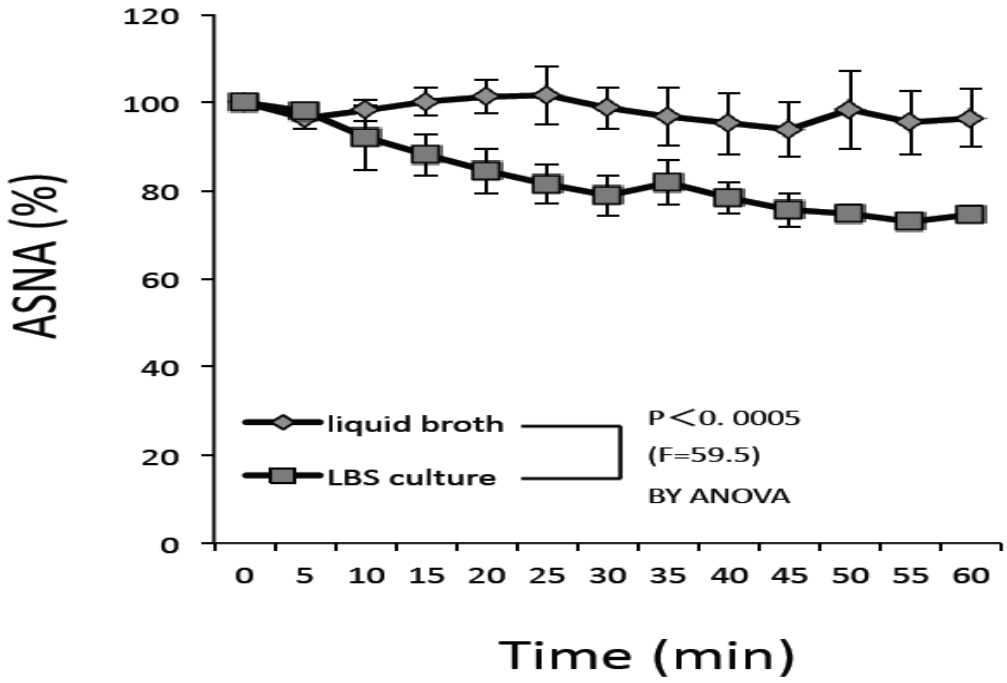
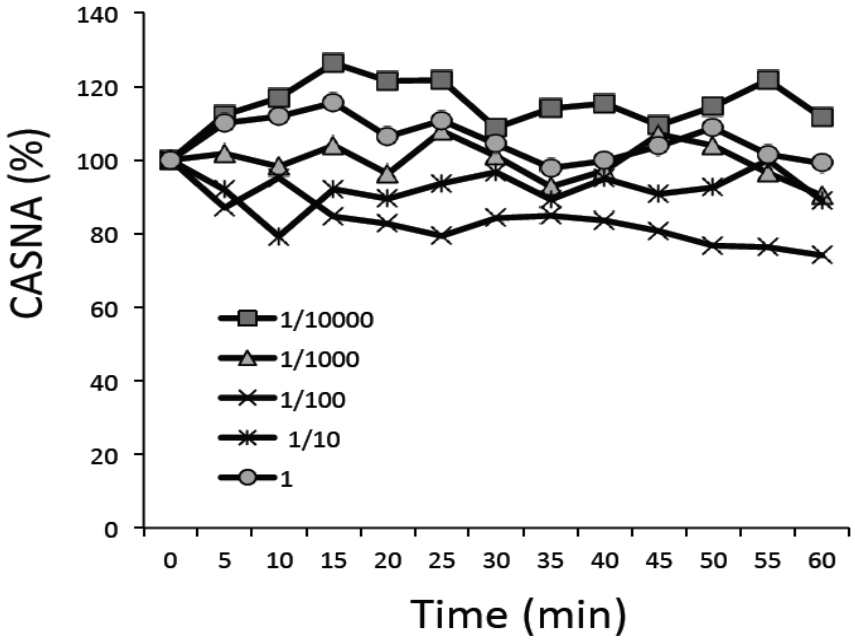

ASNA (%)
120
100
80
60
40
20
0
liquid broth
LBS culture
P＜0. 0005
(F=59.5)
BY ANOVA
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (min)

Fig. 2

(A)


CASNA (%)
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120
100
80
60
40
20
0
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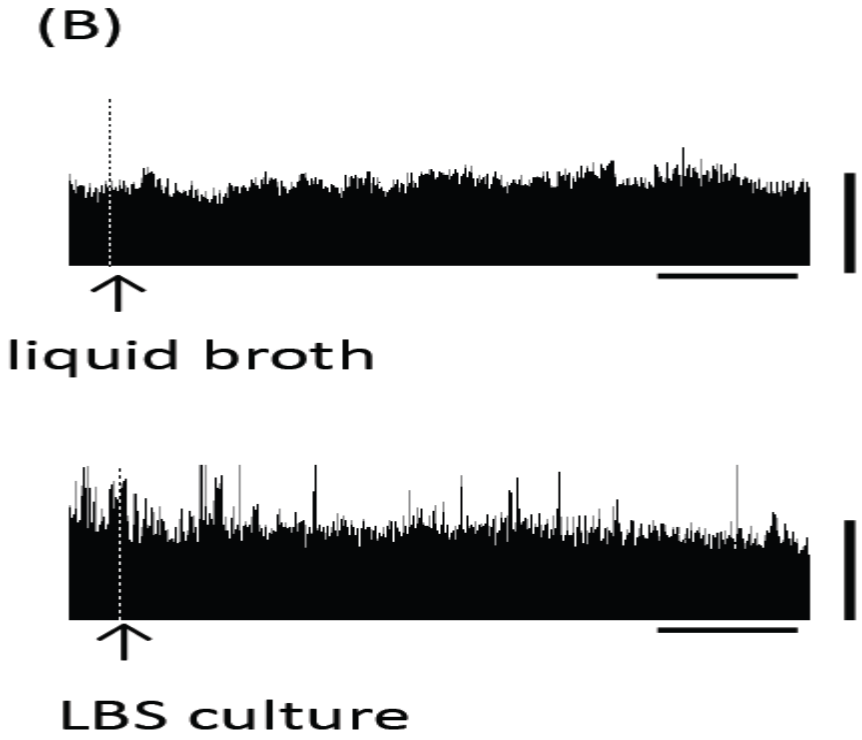
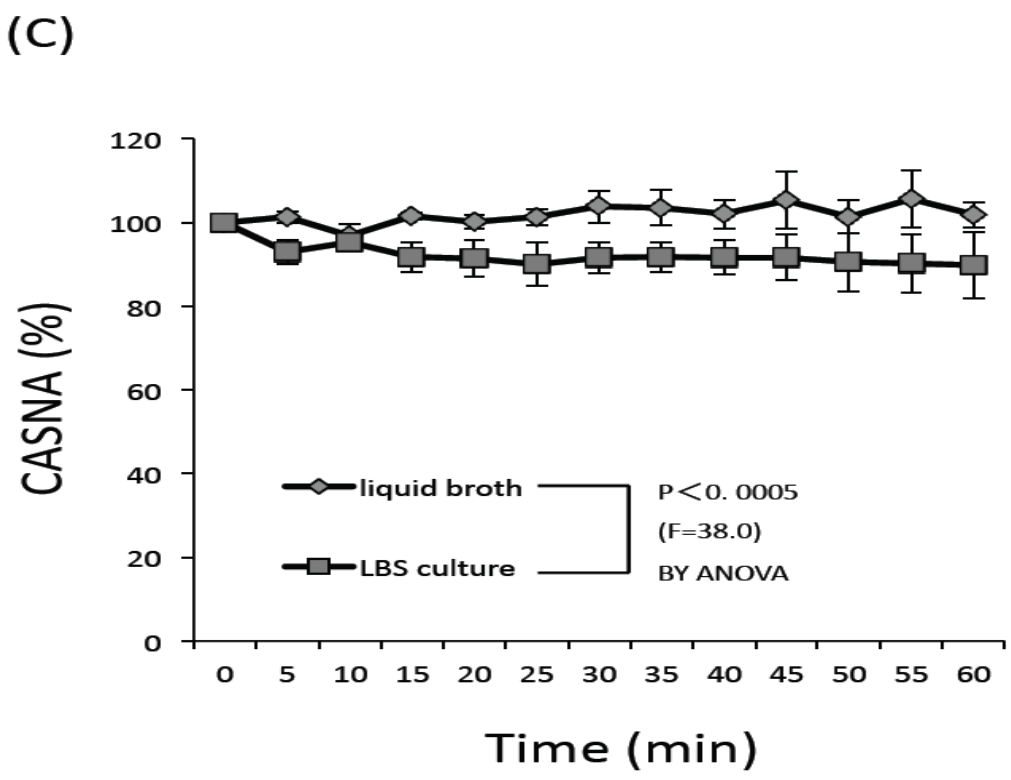
1/1000
1/100
1/10
1
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (min)


(B)
liquid broth
LBS culture


(C)
CASNA (%)
120
100
80
60
40
20
0
liquid broth
LBS culture
P＜0. 0005
(F=38.0)
BY ANOVA
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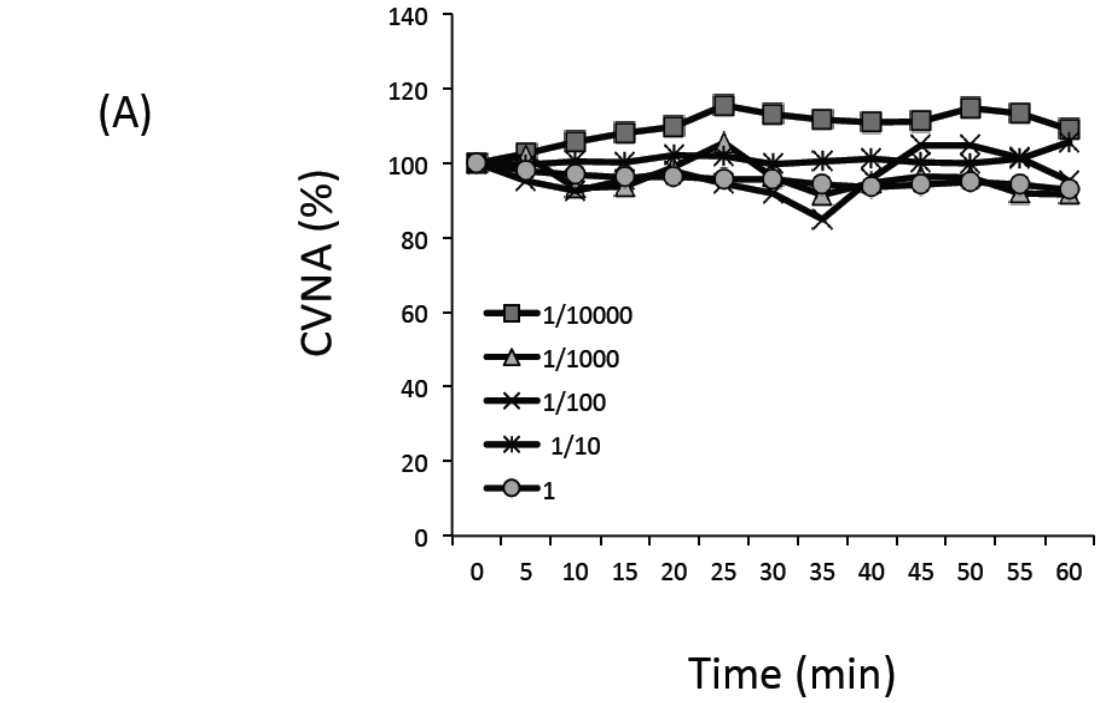
5
10
15
20
25
30
35
40
45
50
55
60
Time (min)

Fig. 3


(A)
CVNA (%)
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120
100
80
60
40
20
0
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Time (min)
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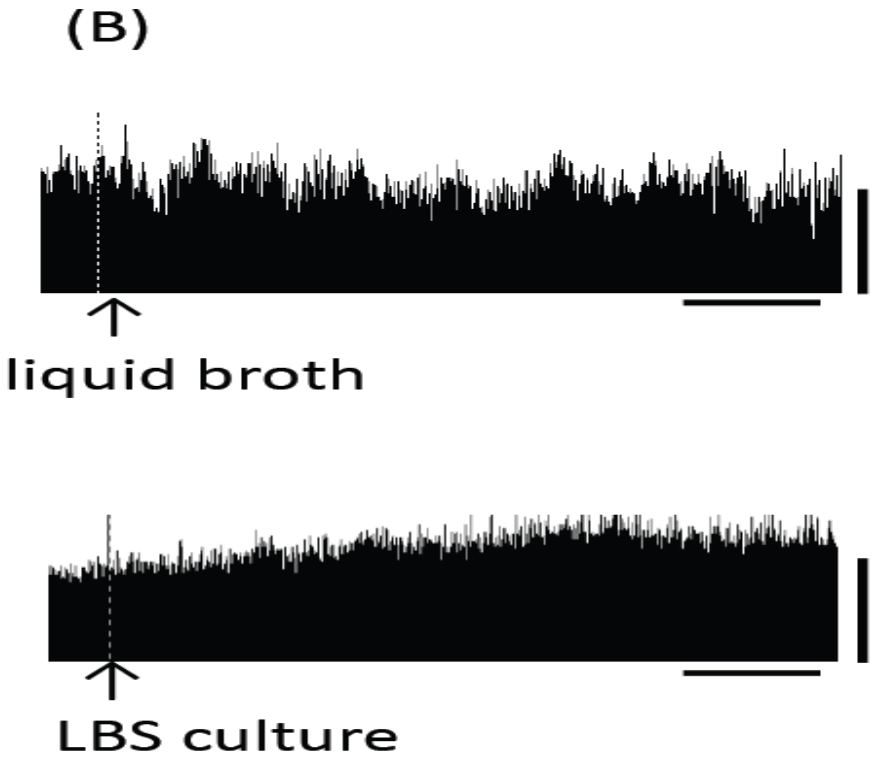
1/1000
1/100
1/10
1


(B)
liquid broth
LBS culture

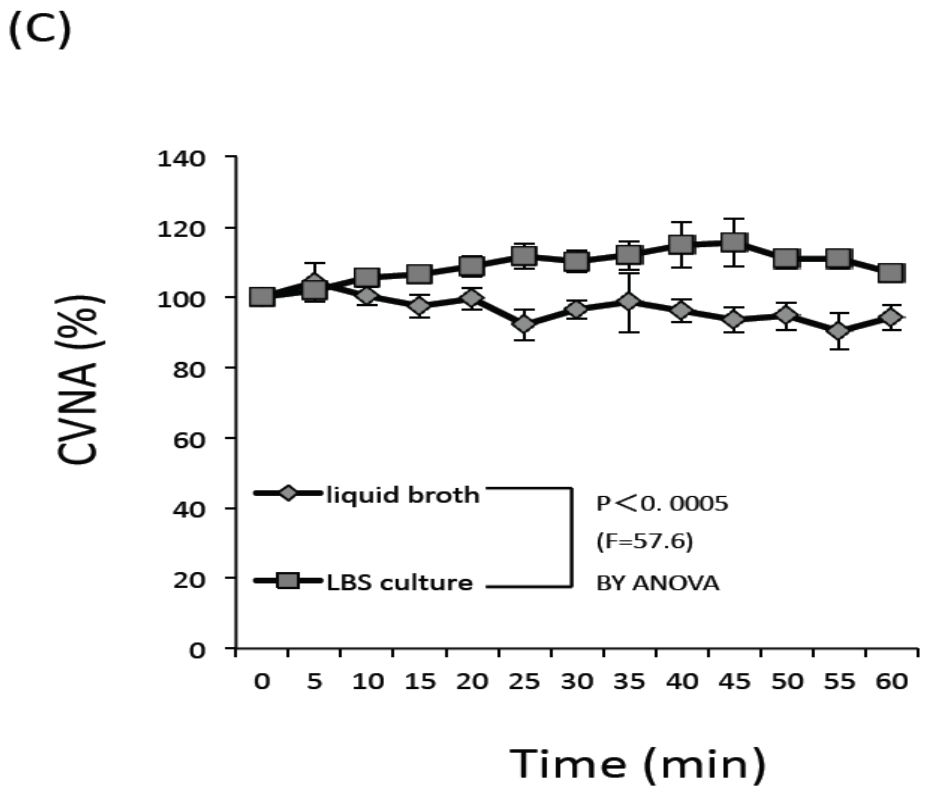


FIGURE LEGENDS

Fig. 1. Effect of dermal application of LBS culture on adrenal sympathetic nerve activity (ASNA) in rats. (A) The changes in neural activity after dermal application of several dose of LBS culture. (B) Representative recordings of ASNA application of liquid broth or 100-fold LBS culture. The horizontal solid bar indicates a time duration of 10 min and the vertical solid bar to the right of the recording represents a neural discharge rate of 200 spikes/5 sec. The arrows indicate the time of injections. (C) Changes in ASNA after application of liquid broth or100-foldLBS culture are expressed as mean ± SEM of the percentages of 0 min values. Significant differences (* = $P < 0.05$) between values from 5 to 60 min after dermal application of liquid broth or 100-fold LBS culture were analyzed by ANOVA with repeated measures.

Fig. 2. Effect of dermal application of LBS culture on cutaneous arterial sympathetic nerve activity (CASNA) in rats. (A) The changes in neural activity after dermal application of several dose of LBS culture. (B) Representative recordings of CASNA application of liquid

broth or 100-fold LBS culture. The horizontal solid bar indicates a time duration of 10 min and the vertical solid bar to the right of the recording represents a neural discharge rate of 200 spikes/5 sec. The arrows indicate the time of injections. (C) Changes in CASNA after application of liquid broth or100-fold LBS culture are expressed as mean ± SEM of the percentages of 0 min values. Significant differences (* = $P < 0.05$) between values from 5 to 60 min after dermal application of liquid broth or 100-fold LBS culture were analyzed by ANOVA with repeated measures.

Fig. 3.Effect of intragastic administration of LBS culture on celiac vagal nerve activity (CVNA) in rats. (A) The changes in neural activity after intragastic administration of several dose of LBS culture. (B) Representative recordings of CVNA application of liquid broth or 10,000-fold LBS culture. The horizontal solid bar indicates a time duration of 10 min and the vertical solid bar to the right of the recording represents a neural discharge rate of 200 spikes/5 sec. The arrows indicate the time of injections. (C) Changes in CVNA after application of liquid broth or10,000-fold LBS culture are expressed as mean ± SEM of the percentages of 0 min values. Significant differences (* = $P < 0.05$) between values from 5 to 60 min after dermal application of liquid broth or 10,000-fold LBS culture were analyzed by ANOVA with repeated measures.