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Note

Oral administration of *Lacticaseibacillus casei* ATCC393 promotes angiogenesis by enhancing neutrophil activity in a murine hind-limb ischemia model

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Angiogenesis is a highly regulated biological event and requires the participation of neutrophils, which are innate immune cells, to initiate the systematic responses. Some strains of lactic acid bacteria (LAB) can be used for probiotics that provide functional modifications in our immune systems. Here, we show that oral administration of *Lacticaseibacillus casei* ATCC393 promoted inflammatory angiogenesis accompanied by enhanced neutrophil activity. Heat-killed *L. casei* (HK-LC) administration improved angiogenesis in a murine hind-limb ischemia (HLI) model. The recruitment and activity of neutrophils were enhanced by HK-LC administration under the HLI conditions. Our results provide novel evidence of an immunological contribution of LAB uptake in the prevention of or recovery from cardiovascular diseases.

Key words: angiogenesis, neutrophil, NETosis, lactic acid bacteria, probiotics

Angiogenesis is an important biological event in healthy individuals as well as in certain conditions, such as disease and injury [1]. In particular, it is a critical event in cardiovascular disease, because the guarantee of sufficient blood flow is an important factor that attenuates tissue injury and promotes the recovery process in wounded tissue [2, 3]. Although the major steps in neovascularization are comprehensively covered by physiological and molecular biological events, the immune system also has an indispensable role [4–6]. Neutrophils, innate immune cells of myeloid lineage, are generally considered to provide the primary defense against pathogenic invasion in our body [7]. However, they have far greater functions than our traditional expectations. In fact, they are involved in inflammatory angiogenesis, which is induced by ischemic injury [8, 9]. The proper recruitment of neutrophils into damaged lesions promotes neovascularization and prevents further expansion of tissue injury due to serious oxygen loss in the tissue [10–12]. Given their natural character, neutrophils release inflammatory mediators, such as cytokines, reactive oxygen species (ROS), anti-microbial peptides (AMPs), myeloperoxidase (MPO), and neutrophil elastase (NE), and these mediators are important in inflammatory angiogenesis [13, 14]. For instance, an increased ROS level in the cytosol is necessary to induce neutrophil extracellular trap (NET) formation, which is characterized by the release of a decondensed chromatin structure, generated in a peptidyl arginine deiminase-4 (PAD-4)-mediated manner, into the extracellular space accompanied by MPO and NE [15, 16]. In general, NET formation is the most dramatic activity in neutrophils against invading pathogens, because the NET structures physically trap the pathogens to eliminate them with enzymes and chemical substances produced by the neutrophils [15, 16]. On the other hand, recent reports have shown that NETosis is an indispensable neutrophil-based response in inflammatory angiogenesis [8, 9]. *In vitro* experiments showed that NETosis induced the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-*k*B) and upregulated intercellular adhesion molecule-1 (ICAM-I) expression, both of which promote angiogenesis, in endothelial cells [8]. In addition, treatment with DNase and an MPO inhibitor suppressed NF-*k*B upregulation in endothelial cells, implying that NET-related factors, such as cell-free DNA (CFD) and MPO, substantially contribute to the promotion of angiogenesis [8]. This evidence suggests that endothelial cells do

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not require direct contact with neutrophils but that some factors released from neutrophils upon NETosis have crucial roles in the promotion of inflammatory angiogenesis.

Innate immunity is frequently modified by internal environmental changes induced by food and supplement intake via the gut [17–19]. In particular, the functional modification of intestinal dendritic cells (DCs) and macrophages has been well documented [20–23]. Probiotics are useful tools for inducing immunological functional modifications not only for the intestinal environment but also in whole body, and the usage of lactic acid bacteria (LAB) is one of the major approaches in probiotics [20–23]. Probiotic-mediated microbiome changes are triggers that induce the functional alteration of myeloid cells, including neutrophils, which implies that biological and microbiological information of the intestinal environment might be transferred to bone marrow (BM) and other immune system-related organs/tissues [24]. Therefore, we were interested in the function of neutrophil, which is modified by probiotic LAB. In this report, we show evidence that *Lacticaseibacillus casei* ATCC393 administration induces a functional modification of neutrophils that consequently promotes inflammatory angiogenesis in a murine hind-limb ischemia (HLI) model.

To investigate the effect of oral administration of *L. casei* ATCC393 on angiogenesis, we established a murine HLI model (Fig. 1A) [25]. C57BL/6 J wild type (WT) mice (8–16 weeks, female, n=5 in each group) received oral administrations (intragastric, i.g.) of heat-killed *L. casei* (HK-LC; 200 µL of 5×10^9 CFU/mL suspension in saline) or saline (200 µL) for 14 days (day −14 to 0). *L. casei* ATCC393 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HK-LC

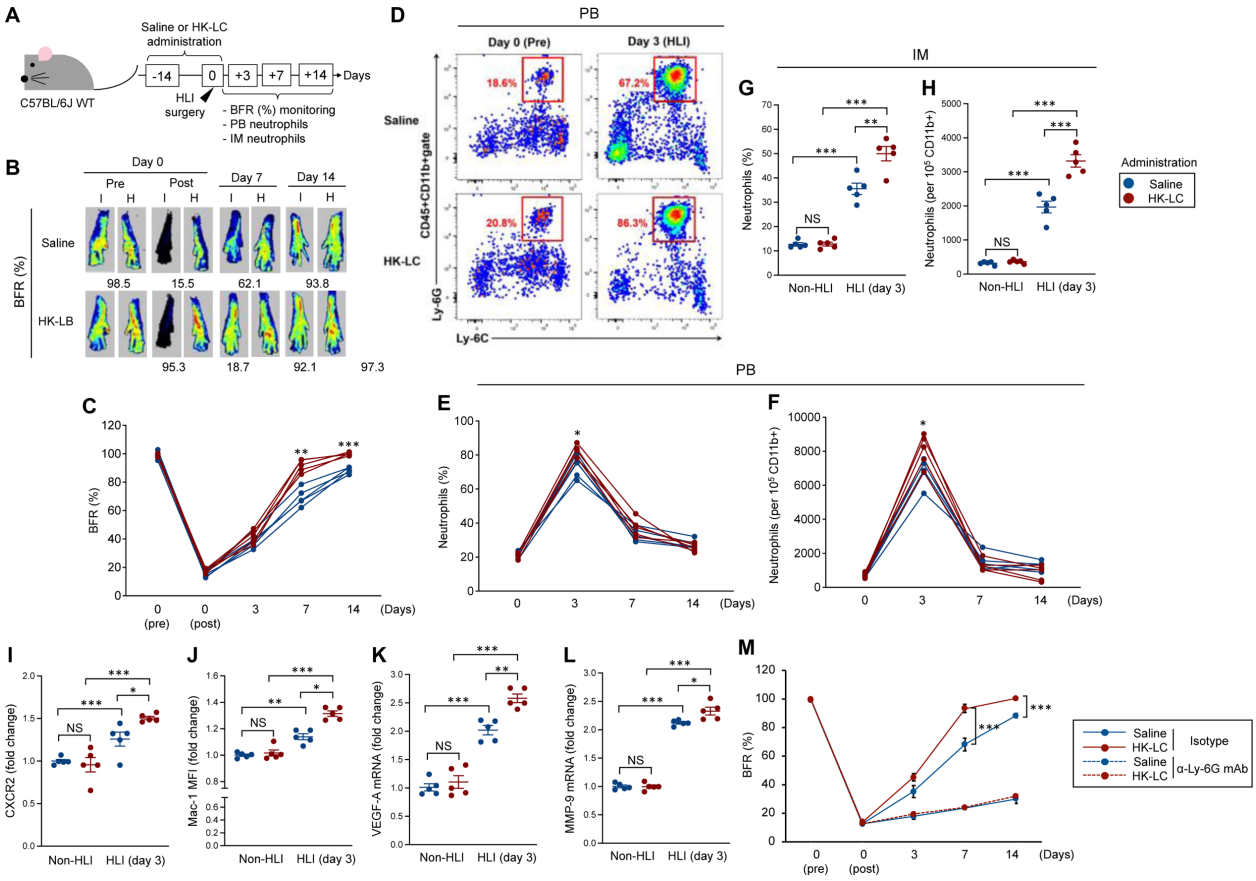

Fig. 1. Promotion of angiogenesis accompanied by increased neutrophil recruitment by oral administration of *L. casei* ATCC393.

A) Study design of heat-killed *L. casei* (HK-LC) administration and the hind-limb ischemia (HLI) model. C57BL6/J WT mice (8–16 weeks, female, n=5 in each group) received oral administrations of saline (200 µL) or HK-LC (200 µL of a 5×10^9 CFU/mL suspension in saline) for 14 days (day −14 to 0), and then an ischemic injury was surgically induced in their left hind limb on day 0. Blood flow was monitored by the laser doppler method, and blood flow rate (BFR) (%) was calculated on days 0 (pre- and post-surgery), +3, +7, and +14. The neutrophil percentage (Ly-6GhiLy-6C+ in the CD45+CD11b+ gate) and number (Ly-6GhiLy-6C+cells per 10^5 of CD45+CD11b+) were also investigated in peripheral blood (PB) by flow cytometry on days 0, +3, +7, and +14. Furthermore, the frequency and number of intramuscular (IM) neutrophils were analyzed by flow cytometry in non-HLI mice (only received oral administration of saline or HK-LC) as well as in HLI mice (day +3). B) Representative images of BFR. C) Transition of BFR. D) Representative images of the neutrophil population in PB on day 0 (pre-surgery) and day +3 (HLI). E, F) The percentage (E) and number (F) of PB neutrophils. G, H) The frequency (G) and number (H) of IM neutrophils. I, J) Expression of CXCR2 and Mac-1 in PB neutrophils. PB samples (day 3 of HLI) were subjected to analysis of the expression levels of CXCR2 (I) and Mac-1 (J) by flow cytometry. K, L) Gene expression of VEGF-A and MMP-9 in neutrophils. PB neutrophils were isolated from the HLI mice (day 3), and total RNA was subjected to real-time PCR to quantify the mRNA expression of VEGF-A (K) and MMP-9 (L). M) Neutrophil depletion in the HLI model. HLI mice generated by following the protocol indicted in (A) received intraperitoneal (i.p.) injections of isotype antibodies or anti-1A8 mAb (100 µg/dose) from day 0 (post-surgery) to +14 every 24 hr. BFR (%) was calculated on days 0 (pre- and post-surgery), +3, +7, and +14. The cumulative data are shown as the mean ± SEM of five samples in two independent experiments. One-way ANOVA was used to analyze data for significant differences. Values of *p<0.05, **p<0.01, and ***p<0.001 were regarded as significant, respectively. NS: non-significant.

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was prepared by heating the bacterial cells at 95°C for 10 min [23]. On day 0, the mice were operated on to induce ischemic injury, and the blood flow rate (BFR) was monitored on days 0 (both pre- and post-surgery), +3, +7, and +14 by laser doppler imaging (LDI) using a PeriCam PSI HR system (Perimed, Las Vegas, NV, USA). BFR (%) was calculated for the ischemic side with the BFR of the healthy side considered 100%. At the same time, blood circulating neutrophils were characterized by flow cytometry using LSR II and FACSCanto systems (BD Biosciences, Franklin Lakes, NJ, USA). All experiment protocols were reviewed and approved by the Animal Welfare Committee of Academia Sinica (protocol No. 15-10-875) and Jichi Medical University (protocol No. 20036-01, 20037-01).

The BFR of the ischemic hind limb was completely inhibited compared with the healthy side in the mice after HLI surgery (day 0 post-surgery). Saline-administered control mice showed BFRs of 38.3 ± 4.92% and 69.38 ± 6.25% on days +3 and +7, respectively. HK-LC-administered mice showed almost the same BFR on day +3 (41.4 ± 4.65%); however, the values were significantly improved in this group on day +7 compared with the control group (91.56 ± 4.42%). On day +14, both groups showed their highest BFRs, and most of the mice showed a BFR of over 85%. However, there was still a significant difference in BFR between the control- and HK-LC-administered groups, the BFRs of which were 88.2 ± 2.16% and 99.9 ± 1.02%, respectively (Fig. 1B, 1C).

Neutrophils are innate immune cells that are driven in the initial stage of pathogenic invasion; however, they also have critical roles in inflammatory angiogenesis [8, 9, 15, 16, 26]. Therefore, we analyzed the recruitment of peripheral blood (PB) neutrophils by flow cytometry. PB was collected from the cervical vein and immediately treated with RBC lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature (RT) for 10 min. After being washed with phosphate buffered saline (PBS), PB leukocytes were treated with an anti-CD16/CD32 monoclonal antibody (mAb; FcγRII/III blocker, clone 2.4G2, Bio Legend, San Diego, CA, USA) at 4°C for 10 min followed by surface staining with a fluorochrome-conjugated mAb in PBS/2% fetal bovine serum (FBS) at 4°C for 30 min. The mAbs used for flow cytometry analysis were anti-CD45 PerCP-Cy5.5 (clone: 30-F11), anti-CD11b Phycoerythrin-Cyanine7 (PE-Cy7; clone: M1/70), Ly-6G Allophycocyanin (APC; clone: 1A8) and Ly-6C Pacific Blue (clone: HK1.4), which were all purchased from BioLegend (San Diego, CA, USA). In this analysis, we found that both the frequency and number of neutrophils, which were determined as Ly-6GhiLy-6C+ (in a CD45+CD11b+ gate), were greatly increased on day +3 compared with day 0 (pre-surgery) and even on day +7 in the control mice. HK-LC-administered mice also showed highly elevated neutrophil frequencies in the peripheral blood on day +3, and the values were significantly larger than those of the control mice (82.0 ± 3.83% for HK-LC vs. 73.1 ± 6.35% for saline; Fig. 1D, 1E). Corresponding to the percentage increase, the numbers of neutrophils were significantly increased in HK-LC-administered mice as compared with those of control mice (8,062 ± 913 vs. 6,803 ± 780 per 10^5 of CD11b+ cells for HK-LC and saline, respectively; Fig. 1F).

We also investigated the population of intramuscular (IM) neutrophils. The muscles (quadriceps femoris, QF; hamstring; gastrocnemius, GC; tibialis anterior, TA) were extracted from each mouse and digested with collagenase I (1 mg/mL; Sigma Aldrich, St Louis, MO, USA) at 37°C for 60 min. The cell suspension was subjected to flow cytometry. Corresponding to the increase in neutrophils in PB, the percentage of IM neutrophils detected with the same gating as in the PB neutrophil analysis was also increased on the ischemic side on day +3 after the ischemic injury was induced compared with the healthy side. In addition, neutrophil recruitment on the affected side was significantly promoted in the HK-LC-administered mice compared with the saline-administered control mice (Fig. 1G, 1H). To investigate the substantial contribution of neutrophils to the promotion of angiogenesis in the HK-LC-administered mice, we investigated the character and activities of neutrophils in HLI mice. The expression levels of C-X-C Motif Chemokine Receptor 2 (CXCR2) and macrophage-1 antigen (Mac-1), which are important cell-surface molecules for chemotaxis and cell adhesion in inflammatory environments, were both significantly upregulated in PB neutrophils of HK-LC-administered mice as compared with controls under HLI conditions (day 3; Fig. 1I, 1J). We also investigated the gene expression levels of angiogenesis-related factors, such as vascular endothelial growth factor A (VEGF-A) and matrix metallopeptidase 9 (MMP-9), in PB neutrophils by real-time polymerase chain reaction (PCR). The expression levels of both genes were significantly upregulated in HK-LC-administered mice compared with those of control mice under HLI conditions (day 3; Fig. 1K, 1L). Furthermore, we depleted the levels of neutrophils and investigated the progress of angiogenesis in the HLI model. Mice that received the HLI surgery received an intraperitoneal (i.p.) injection of 100 μg of isotype antibody or anti-Ly-6G mAb (*InVivo*MAb, Bio X Cell, Lebanon, NH, USA) every 24 hr from day 0 (post-surgery) to day +14, and then BFRs were monitored in the mice. The depletion of neutrophils obviously suppressed angiogenesis compared with the isotype antibody-treated mice. There was no difference in the tendencies of BFR between the saline- and HK-LC-administered mice with neutrophil depletion (Fig. 1M).

Next, we investigated the effect of HK-LC administration on neutrophil activity. In this study, we arranged for two different groups of mice: non-HLI and HLI mice. Mice in both groups were treated with HK-LC or saline administration for 14 days (Fig. 2A). We first measured NETosis-related factors, such as CFD, myeloperoxidase-DNA complex (MPO-DNA), and citrullinated histone H3 (CitH3), which are released into the blood stream from NETs formed by neutrophils [27–29]. Serum samples were obtained from PB of both groups on day +3, and the contained CFD was stained with SYTOX™ Green at 37°C for 15 min followed by detection of fluorescence using a Spark microplate reader (Tecan, Maennedorf, Switzerland). The serum concentration of CFD was calculated by the standard curve method [30]. MPO-DNA was measured in the serum by a protocol described in a previous report, with minor modifications [30]. Briefly, MPO was captured with the capture antibody in a Human Myeloperoxidase enzyme-linked immunosorbent assay (ELISA) Kit (Abcam, Cambridge, UK), and then the MPO-bound DNA was detected with the detection antibody in a Cell Death Detection ELISA kit (Roche, Basel, Switzerland). CitH3 was measured by using a Mouse Citrullinated Histone H3 ELISA Kit (MyBioSource, San Diego, CA, USA). The levels of these three factors were comparable between saline- and HK-LC-administered mice without ischemic injury. However, all of these values were significantly increased in the HK-LC-administered

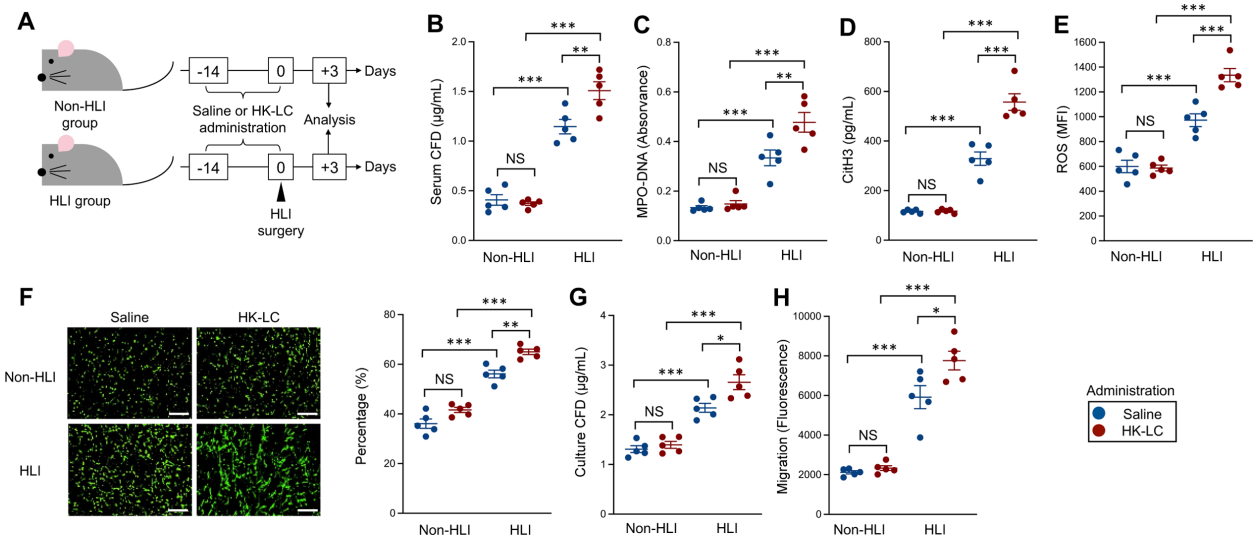

Fig. 2. Enhanced neutrophil activity in *L. casei* ATCC393-administered mice under ischemic injury.

A) Study design for the *in vitro* neutrophil activity assay. The mice received saline or heat-killed *L. casei* (HK-LC) administration for 14 days, and then the mice in the hind-limb ischemia (HLI) group were operated on to induce an ischemic injury on day 0. Blood was collected from the non-HLI and HLI mice on day +3, and then serum and neutrophils were isolated and used in subsequent assays. B–D) Serum samples were used to measure cell-free DNA (CFD) (B), myelope roxidase (MPO)-DNA (C), and CitH3 (D). E) Reactive oxygen species (ROS) production in neutrophils. Neutrophils isolated from blood were stained with CellROX^{TM} Green at 37°C for 60 min and then analyzed by flow cytometry. F, G) NETosis of neutrophils. Neutrophils isolated from blood were seeded in a 96-well flat-bottom plate in the presence of PMA (50 nM). After incubation at 37°C for 4 hr, the cells were stained with SYTOX^{TM} Green for microscopic analysis (F). The cultured medium was collected, and CFD was stained with SYTOX^{TM} Green followed by measurement of the fluorescence signal using a microplate reader (G). H) Migration ability of neutrophils. Neutrophils isolated from blood were stained with carboxyfluorescein succinimidyl ester (CFSE) and seeded in a transmigration well (3 μm), and then the well was placed in a 96-well flat-bottom plate applied with CXCL1 (1 μg/mL) containing RPMI complete medium. After 120 min, the fluorescence signal from migrated cells was measured by microplate reader. The cumulative data are shown as the mean ± SEM of five samples in two independent experiments. One-way ANOVA was used to analyze data for significant differences. Values of *p<0.05, **p<0.01, and ***p<0.001 were regarded as significant, respectively. NS: non-significant.

mice compared with the saline-administered mice under the HLI conditions (Fig. 2B–2D).

Next, we investigated the cellular activity of neutrophils. Neutrophils were isolated from blood, which was collected by cardiac puncture, using an EasySep^{TM} Mouse Neutrophil Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada) and then used for ROS production and NETosis assays. The purity of the neutrophils was analyzed in each experiment, and CD11b+Ly-6GhiLy-6C+ population was confirmed to comprise over 90% of each sample.

Neutrophils (1.0 × 10^6/mL) were treated with CellROX^{TM} Green (500 nM) at 37°C for 60 min, and then flow cytometry was performed by the identical gating strategy and surface staining described in Fig. 1. For the NETosis assay, neutrophils (5.0 × 10^5/mL) were seeded in a 96-well plat bottom plate (poly-L-lysine coated) with phorbol 12-myristate 13-acetate (PMA; 50 nM, Sigma Aldrich, St. Louis, MO, USA) containing RPMI complete medium (RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin) and then incubated at 37°C for 4 hr. NET formation was observed by fluorescence microscope (BZ-X700, Keyence, Osaka, Japan), and CFD in the cultured medium was measured by the method used in Fig. 2B. ROS production was significantly increased in neutrophils from HK-LC-administered mice compared with those from saline-administered mice under the HLI conditions. However, the ROS production of the neutrophils was comparable in the two different administration groups under the non-HLI conditions (Fig. 2E). The average percentage of NETosis cells was slightly increased in the HK-LC-administered mice compared with the saline-administered mice without ischemic injury, but the difference was not significant. The percentages of NETosis cells were greatly increased in both administration groups of mice under the HLI conditions, and that for HK-LC-administered mice was significantly increased compared with that for saline-administered mice (65.0 ± 2.48% for HK-LC vs. 56.1 ± 3.43% for saline; Fig. 2F). The CFD concentration was also significantly increase in the HK-LC-administered mice compared with the saline-administered mice under the HLI conditions. On the other hand, the CFD level was comparable between two administration groups under the non-HLI conditions (Fig. 2G). PB-isolated neutrophils were also used for a migration assay. Neutrophils (1.0 × 10^6/mL) were first stained with carboxyfluorescein succinimidyl ester (CFSE) at 37°C for 30 min and then seeded in a transmigration well (pore size 3 μm), and the well was then placed in a 96-well flat-bottom plate applied RPMI complete medium containing chemokine (C-X-C motif) ligand 1 (CXCL1, 1 μg/mL). The culture was incubated at 37°C for 120 min, and then the fluorescence originating from the migrated neutrophils in the bottom well was measured by Spark microplate reader (Tecan). The migratory activity of the neutrophils was comparable between saline- and HK-LC-administered mice without ischemic injury. On the other hand, the neutrophils isolated from the HLI mice showed significantly increased migration in those administered HK-LC compared with those administered saline (Fig. 2H).

Our data suggested that *L. casei* ATCC393 administration promotes inflammatory angiogenesis in ischemic injury

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accompanied by functional enhancement of neutrophils. *L. casei* ATCC393 has been reported to possess a probiotic function. Oral administration of *L. casei* ATCC393 attenuated endotoxin-induced intestinal barrier dysfunction by regulating Toll-like receptor expression in intestinal epithelial cells [31]. Moreover, intestinal tissue damage in dextran sulfate sodium (DSS)-induced colitis was attenuated in *L. casei* ATCC393-administered mice by suppressing NLR family pyrin domain containing 3 (NLRP3) activation and pro-inflammatory cytokine production in the colon [32].

On the other hand, functional modification of neutrophils has yet to be reported. HK-LC administration promoted neutrophil recruitment and functional upregulation in the mice under physiological conditions, and this modification established a substantial environmental change that promoted angiogenesis when the mice received the ischemic injury (Fig. 1). Although the basal neutrophil status was not changed under the healthy conditions (non-HLI), the total activity of the neutrophils was significantly enhanced by HK-LC administration under the ischemic injury conditions. Therefore, we hypothesized that the HK-LC-mediated functional modification in the neutrophils expanded and became more obvious under the physiologically stressed conditions (Fig. 2). Since neutrophils have fast turnover compared with other immune cells and their generation/development is based on BM [33, 34], we suspected that an *L. casei* ATCC393 signal was transferred from the gut to the BM that induced the production of neutrophils with potentially enhanced function. The 14 days of administration of HK-LC seemed to provide a sufficient effect in the mice to induce an environment change in the BM, especially for neutrophil differentiation and maturation. Providing clear evidence of gut-BM cross talk in neutrophil development is important in the fields of both probiotics and immunology studies. Although we did not investigate the mechanisms of this phenomenon in the current study, we intend to take on the challenge of revealing the detailed mechanisms with some of the latest approaches, such as epigenetics and metabolomics, in future studies. Other immune cells are also involved in the inflammatory angiogenesis process; however, we did not investigate them in this study [35]. To understand the *L. casei* ATCC393-derived immunological environment changes in inflammatory angiogenesis, other immune cells generally involved in angiogenesis, such as macrophages and T cells, must be investigated together. In fact, we have already reported that HK-LAB supplementation activated DCs, macrophages, and T cells in mice [20–23]. In the present study, we also did not perform a detailed investigation on the mechanistic parts of the neutrophil activation induced by *L. casei* ATCC393 administration. For instance, the sensing receptors and molecular mechanisms in the activation of *L. casei* ATCC393-exposed neutrophils must be understood; however, we have not described them yet. These parts need to be revealed in future studies, and the detailed evidence this provides will increase the possibility of utilizing *L. casei* ATCC393 as a probiotic strain targeting the functional modification of neutrophils.

Although LAB and probiotics are generally understood to provide only positive effects on our immune systems, we must carefully consider their effects, especially for neutrophil-related events. Neutrophil overactivation is a possible cause of aggravation of septic shock and tumor metastasis [26, 36]. Aggressive pro-inflammatory cytokine production in neutrophils is considered to cause expanded and prolonged inflammation [26]. Notably, promoted NET formation has been reported as a critical factor in the expansion of tumor metastasis in certain types of tumors by induction of genetic and morphologic instability in the tumor cells [37].

Therefore, the adoption of L. casei in probiotics might need to be considered well depending on the situation.

Once a disease condition becomes critical, LAB used for probiotic approaches may have a risk related to disease progression. However, there are obviously positive immune-modifying effects of LAB-mediated probiotics to target that maintain immunological homeostasis under healthy conditions.

AUTHOR CONTRIBUTIONS

The study concept was established by S.S. All of the experiments were performed by S.S. and M.K. Protocols were established by S.S. Data were analyzed and finalized by S.S. and M.K. The manuscript was written by S.S. and M.K. Review and editing were performed by S.S. and M.K. This study was supervised by S.S.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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