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Full Paper

Establishment of porcine fecal-derived *ex vivo* microbial communities to evaluate the impact of livestock feed on gut microbiome

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Sustainable livestock production requires reducing competition for food and feed resources and increasing the utilization of food by-products in livestock feed. This study describes the establishment of an anaerobic batch culture model to simulate pig microbiota and evaluate the effects of a food by-product, wakame seaweed stalks, on *ex vivo* microbial communities. We selected one of the nine media to support the growth of a bacterial community most similar in composition and diversity to that observed in pig donor feces. Supplementation with wakame altered the microbial profile and short-chain fatty acid composition in the *ex vivo* model, and a similar trajectory was observed in the *in vivo* pig experimental validation. Notably, the presence of wakame increased the abundance of *Lactobacillus* species, which may have been due to cross-feeding with *Bacteroides*. These results suggest the potential of wakame as a livestock feed capable of modulating the pig microbiome. Collectively, this study highlights the ability to estimate the microbiome changes that occur when pigs are fed a specific feed using an *ex vivo* culture model.

Key words: gut microbiome, wakame seaweed, culture model, *ex vivo*, pig, fecal fermentation

INTRODUCTION

Livestock production contributes substantially to global environmental changes, particularly in areas with high livestock population densities [1, 2]. This is attributed to associated greenhouse gas emissions, water pollution, acidification, and primary energy consumption, which can have serious impacts on the environment [3, 4]. Feed production, direct energy use, and emissions from housing and manure management systems during pig farming are the major contributors to environmental

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pollution [5]. The fact that the crop production process for feed ingredients relies on fertilizers and agrochemicals, which cause land degradation and transformation and consume large amounts of energy, is also a contributing factor [6, 7]. The solution to this challenge is to mitigate emissions and foster the exploration of alternative resources.

To improve resource use efficiency, reduce food and feed competition, and enhance the cyclicality of the food system, increasing the use of by-products from the food system and inedible parts of foods as livestock feed has been proposed [8]. The use of food system by-products and inedible foods as feed can reduce the environmental footprint of the food system by decreasing greenhouse gas emissions and fertilizer application. Additionally, many by-products and crop residues are readily available as low-cost materials, increasing their utilization as feed and making them cost effective [3].

The gut microbiota is a complex system that plays an important role in health and immunity in pigs [9–13]. It comprises a diverse population of bacteria and other microorganisms [14, 15], and its composition and quantitative and qualitative variations are influenced by both environmental and host genetic factors [16–20]. Previous studies demonstrated a link between microbial profiles and nutritional and productivity parameters [21]. Nutrient and fiber sources in feed have a remarkable impact on the composition of the pig gut microbiota; hence, they must be monitored as part of feeding management [22–24]. However, prior to incorporating food system by-products and inedible foods into livestock feed, assessing their effects on the gut microbiota is crucial.

The brown seaweed *Undaria pinnatifida*, commonly known as wakame seaweed (wakame) is widely cultivated and consumed in East Asian countries. Wakame contains a variety of compounds that exert bioactive functions such as anti-inflammatory, antitumor, and anti-angiogenic activities [25, 26]. Its blades are its main edible parts, and its stalk and roots are discarded during processing. The annual harvest of wakame in Japan is 43,972 tons (Ministry of Agriculture Forestry and Fisheries of Japan, 2021), and more than 70% of the stalks and roots are discarded. Therefore, proper disposal of this biomass (wakame waste) is important for the preservation of coastal ecosystems and the utilization of this organic matter.

Recently, we attempted to use powdered wakame stalks as a pig-feed component. Notably, pigs fed wakame-supplemented feed had a higher ratio of peripheral blood natural killer cells and showed changes in the expression of cytokines and toll-like receptors in small intestinal Peyer's patches [27]. In addition, wakame considerably increased the abundance of *Lactobacillus* and decreased the abundance of *Enterobacteriaceae* in the treatment group, suggesting that wakame can improve gut homeostasis in pigs, either directly or indirectly, by modulating the microbiome [11, 28]. These results encouraged the use of wakame as a livestock feed, which could promote the use of an inedible resource, reduce waste, and provide added value for improving livestock health.

In vitro gut modeling provides a useful platform for the rapid and reproducible prediction of changes in and impacts on the gut microbiome related to foods [29–32], drugs [33], and biocontrol agents [34]. In addition, it provides a host-independent opportunity to investigate the complexity of the gut microbiota and the functional relevance of specific substrates. Several *in vitro* pig gut models have employed batch or continuous cultures to evaluate the fermentative capacity of the gut ecosystems for specific substrates [35–41]. Indeed, these culture systems simulate the gut microbiota composition in the culture vessel such that they resemble that of feces and can be used to monitor changes in short-chain fatty acids (SCFAs) and gut microbiota with the addition of dietary fiber and by-products. However, a notable challenge arises from the limited discourse on the alignment of these changes with diverse *in vivo* parameters.

To address this knowledge gap, we established an *ex vivo* pig microbial community by optimizing bacterial culture media. Furthermore, we implemented a workflow to perform a comparative analysis between *ex vivo* and *in vivo* conditions to analyze the dynamic effects of by-products (i.e., wakame stalks) utilized for livestock feed on the pig microbiome.

MATERIALS AND METHODS

Animals and fecal sample collection

Nineteen pigs at the Miyagi Prefectural Livestock Experiment Station (Miyagi, Japan) were used in this study. Three pigs were 12 weeks old (Landrace, 2 males and 1 female), and the other 16 pigs were 4 weeks old (Landrace, 10 males and 6 females). The three 12-week-old pigs were utilized for culture medium screening in the development of the *ex vivo* model (Fig. 1), while the 16 four-week-old pigs were used in the *in vivo* wakame feeding trial. Eight of the 16 pigs were used as the control group at 14 weeks of age for *ex vivo* cultivation with the supplementation of wakame (Fig. 2). The pigs were housed in a conventional pig barn with ad libitum access to food and water. The diets provided to the pigs were adjusted to contain appropriate levels of crude protein, energy, minerals, and vitamins, according to the recommendations of the National Research Council (NRC, 2012) for the respective growth stages. Pigs were managed according to the Miyagi Prefecture Animal Industry Experiment Station and Miyagi University approved protocols, "R3-2-2(4)" and "No. 2016-23"s, respectively. None of the pigs had received antibiotics for three months leading up to fecal sample collection, and they did not show any signs of disease.

Wakame powder preparation

The wakame stalks were dried for a period of three weeks and subsequently finely ground into a powder. The prepared wakame sample was then stored at a temperature of −20°C until further experiments. A chemical composition analysis of the sample was performed by Japan Food Research Laboratories (JFRL, Tokyo, Japan), and the results are presented in Supplementary Table 1.

Ex vivo model

(i) Fecal sample preparation

One gram of the collected feces from each four-week-old pigs of control groups was suspended in 9 mL of PreserWell MPR (Funakoshi, Tokyo, Japan), a lyophilized protectant for anaerobic bacteria. The fecal suspension was then stored at −80°C until use. Prior to use, the fecal suspension was thawed for 90 sec at 37°C. Subsequently, it was centrifuged at 1,000 rpm for 60 sec, and the supernatant was used for fecal fermentation.

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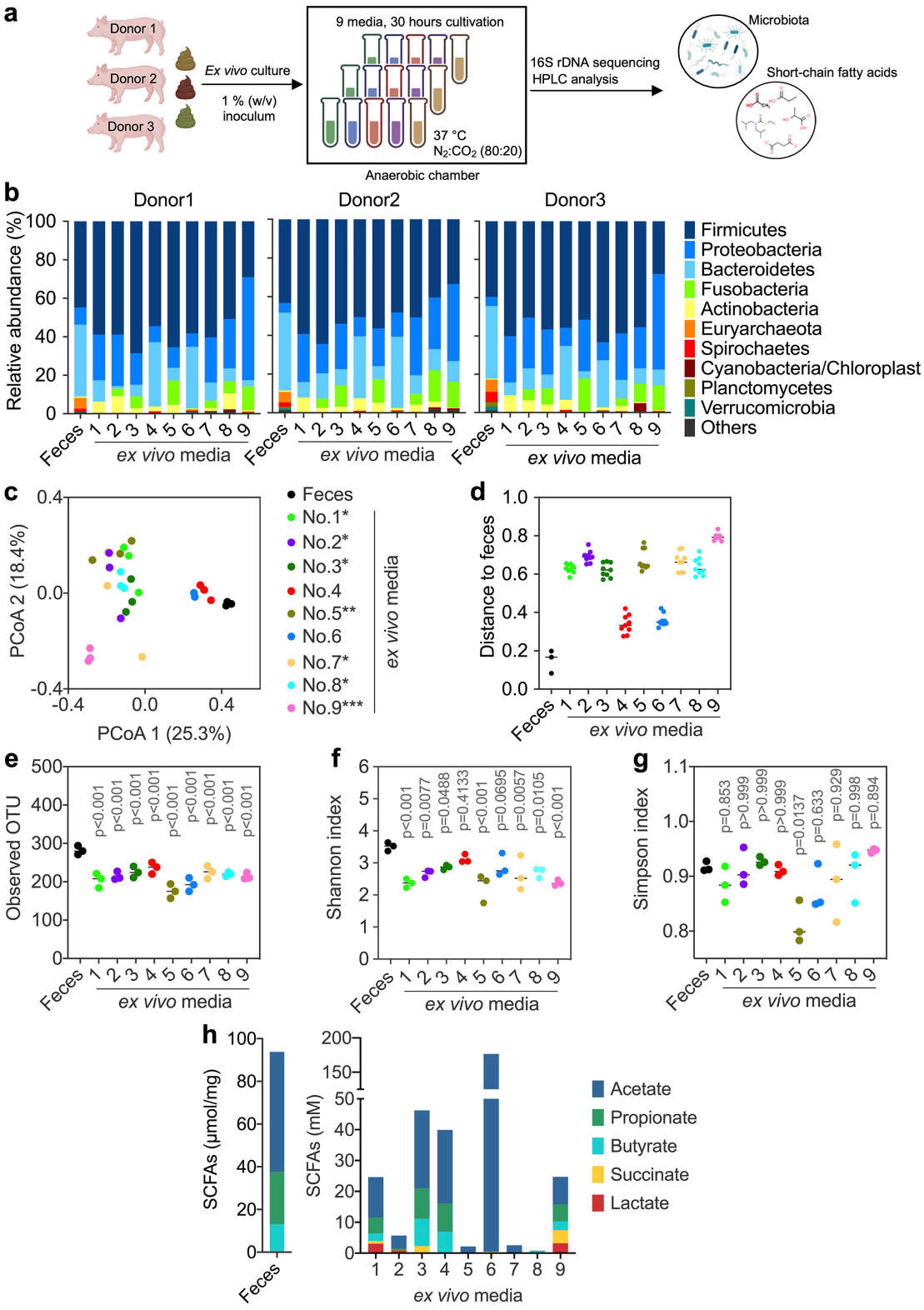

Fig. 1. Screening of *ex vivo* culturing media for supporting the growth of diverse species in the pig fecal microbiome.

(A) Schematic representation of the media selection procedure. (B) Phylum-level bacterial compositions of the donor fecal samples (far left, donors 1–3), as well as those of *ex vivo* cultures, grown anaerobically in nine different media. See Supplementary Table 2 for media compositions. 16S rRNA gene sequences that could not be classified at the phylum level and phyla with less than 1% relative abundance in all samples are grouped into "other". (C) PCoA based on Bray–Curtis dissimilarity for genus-level microbiome communities in the feces (donors 1–3) and media (Nos. 1–9). PERMANOVA was carried out between donor feces and individual media (*p<0.05, **p<0.01, ***p<0.001). (D) *Ex vivo* media microbiota dispersions were assessed based on Bray-Curtis distances from donor feces. (E–G) Observed OTU richnesses, Shannon indices, and Simpson indices of media and donors were plotted, and comparisons were made with one-way ANOVA followed by Dunnett's multiple comparisons. (H) Short-chain fatty acids concentrations in the three donor feces and nine media.

PCoA: principal coordinate analysis; OTU: operational taxonomic unit; SCFAs: short-chain fatty acids; PERMANOVA: permutational multivariate analysis of variance.

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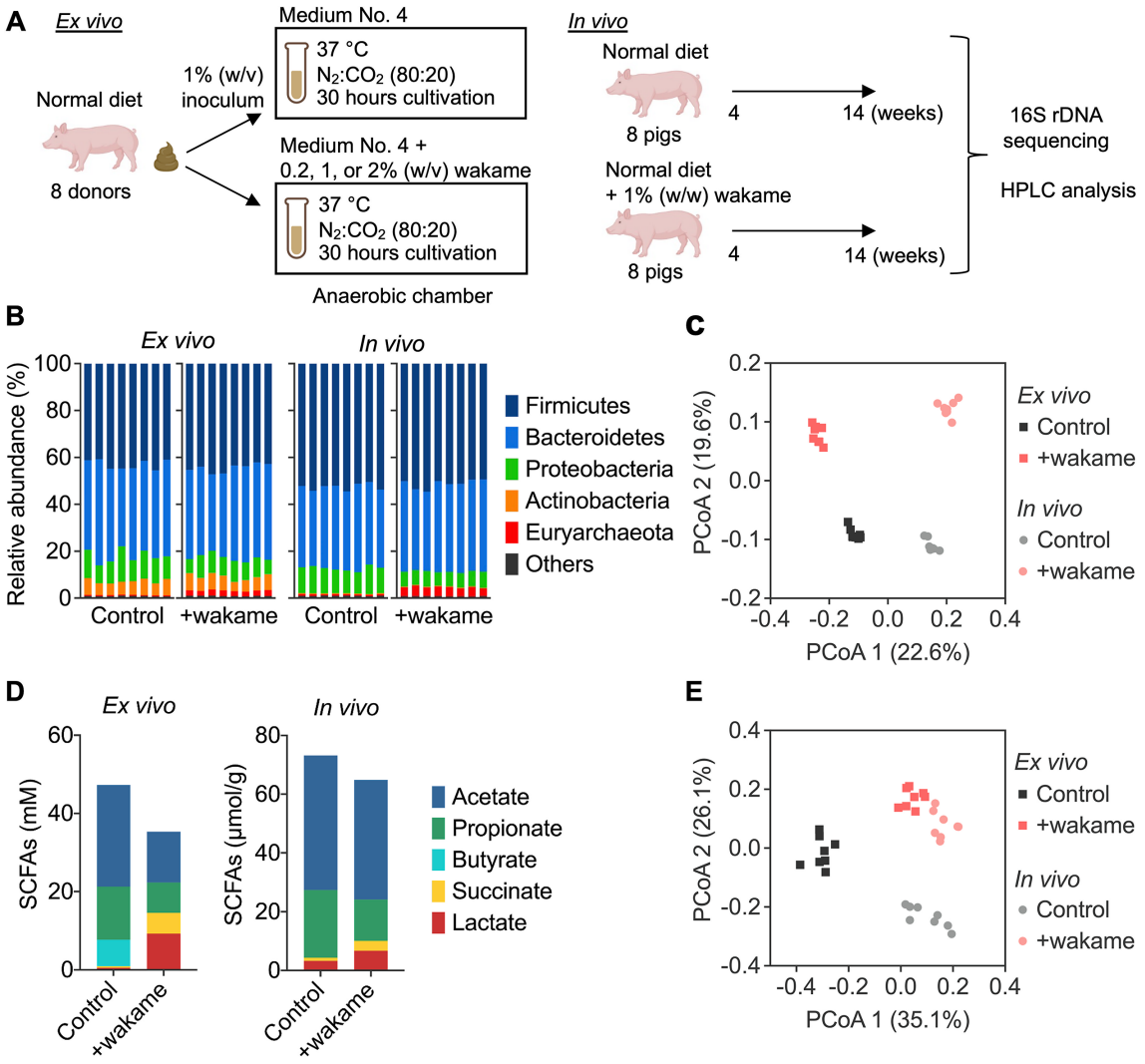

Fig. 2. Microbiome and SCFAs composition changes in response to wakame consumption.

(A) Schematic representation of the *ex vivo* and *in vivo* experiments. (B) Phylum-level bacterial compositions of the *ex vivo* and *in vivo* samples. 16S rRNA gene sequences that could not be classified at the phylum level and phyla with less than 1% relative abundance in all samples are grouped into "other". (C) PCoA based on Bray-Curtis dissimilarity for genus-level microbiome communities of the *ex vivo* and *in vivo* samples. (D) SCFA concentrations in the *ex vivo* and *in vivo* samples. See Supplementary Fig. 4A for a statistical analysis using Student's t-test. (E) PCoA based on Bray–Curtis dissimilarity for the SCFA abundances of the *ex vivo* and *in vivo* samples. See Supplementary Fig. 3B and 3C for a statistical analysis using PERMANOVA.

PCoA: principal coordinate analysis; SCFAs: short-chain fatty acids; HPLC: high-performance liquid chromatography; PERMANOVA: permutational multivariate analysis of variance.

(ii) Media screening

For the screening of culture media in the development of the *ex vivo* model, nine different media (designated as media No. 1 to No. 9) that were previously reported by other researchers for human and pig fecal cultures and used with the aim of reproducing the original bacterial communities were modified by excluding sugar sources (Supplementary Table 2). Each medium was sterilized using an autoclave (121°C, 15 min) and kept in a Bactron Anaerobic Chamber (Shel Lab, Sheldon Manufacturing, Cornelius, OR, USA; atmosphere of 80% N_2, 20% CO_2) overnight to achieve anaerobic conditions. Next, 10 µL of fecal supernatant, diluted 10-fold, was added to 5 mL of each medium, and the cultures were incubated at 37°C for 30 hr. After incubation, 1 mL of the culture medium was aliquoted and stored at −20°C for further analysis.

(iii) Ex vivo cultivation with the addition of wakame

One gram of wakame powder was suspended in 10 mL of distilled water and sterilized by sterile filtration (Millex® syringe filter, 0.45 µm, Merck Millipore, Darmstadt, Germany) and autoclaving (121°C, 15 min). The wakame solution was added to each medium at final concentrations ranging from 0.2% to 2% (w/v). Prior to use, all media were stored overnight in an anaerobic chamber (80% N_2 and 20% CO_2) to achieve anaerobic conditions. Then, 10 µL of the prepared fecal supernatant was added to 5 mL of each medium and subsequently incubated in an anaerobic chamber at 37°C for 30 hr. After incubation, 1 mL of each medium was collected and stored at −20°C.

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In vivo wakame feeding trial in pigs

After an adaptation period with basic feed, the four-week-old Landrace pigs were divided into two groups: i) a control group (*n*=8; consisting of three females and five males) and ii) wakame-fed group (*n*=8; consisting of three females and five males). The control group was fed a basic commercial diet (Kitanihon Kumiai Feed Co., Ltd., Miyagi, Japan). The wakame-fed group, on the other hand, was fed the basic diet supplemented with 1% (w/w) prepared wakame powder for a period of 14 weeks.

16S rRNA gene sequencing

Genomic DNA was subjected to 16S rRNA analysis, and read counts were calculated. The V3–V4 region of the bacterial 16S rRNA gene was amplified using S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGTATCTAATCC-3′) [42]. A 16S rRNA gene sequencing library was prepared according to the manufacturer's instructions (Illumina, San Diego, CA, USA). To normalize the DNA amplicons, the DNA concentrations of polymerase chain reaction (PCR) products were measured using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Appropriate amounts of 16S rRNA gene products and internal controls (PhiX control V3; Illumina, Tokyo, Japan) were subjected to paired-end sequencing on a MiSeq sequencer using a 600-cycle MiSeq reagent kit (Illumina). The paired raw fastq data were merged and quality filtered. The resulting 16S rRNA gene amplicon reads were binned into operational taxonomic units (OTUs) with 97% sequence similarity. The OTUs were taxonomically classified using the Greengenes reference database (v13.8, Illumina) and Ribosomal Database Project (RDP) Classifier [43].

The OTU data were scaled to the minimum number of total reads for each sample type and filtered to remove OTUs present in <100 reads. As an alternative to rarefaction of the data, the data were scaled by dividing each OTU count by the sample total OTU count and by the minimum total OTU counts across samples to normalize the counts to equal depths. Alpha diversity indices (the observed number of OTUs per normalized sample as well as the Shannon and Simpson indices) and beta dispersion estimates were then calculated using the Adonis2 and beta permutation functions of the Vegan package in R, each with 999 permutations [44]. The Adonis2 function performed a PERMANOVA in Vegan on a Bray–Curtis dissimilarity matrix, and the betadisper function assessed the homogeneity of dispersion among groups. Significant differences in microbiota structure between the two groups were evaluated by nonparametric analysis of similarity (ANOSIM) using the Vegan package in R. PCoA plots were generated using the OTU data, and the intersample distance in the distance/dissimilarity matrix was calculated in the R project and plotted using GraphPad Prism (version 9.5.1).

Porcine intestinal epithelial (PIE) #2 cells

PIE #2 cells, which are non-transformed cultured intestinal cells originally derived from the intestinal epithelia isolated from an unsuckled neonatal pig, were kindly provided by the National Institute of Animal Health, National Agriculture and Food Research Organization (article in preparation). PIE #2 cells were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal calf serum, 50 units/mL penicillin, 50 µg/mL streptomycin, and 5 ng/mL epidermal growth factor at 37°C in an atmosphere of 5% CO_2. We used PIE #2 cells between the 7th and 15th passages in these experiments.

Bacterial cells

Ligilactobacillus ruminis (formerly *Lactobacillus ruminis*) JCM 1152^T and *Bacteroides acidifaciens* JCM 10556^T were obtained from the RIKEN BioResource Research Center (Tsukuba, Japan). *L. ruminis* strains 16-7, 425, 450, and 642 were isolated from fecal or milk samples of a sow (the 16S rRNA gene sequence is shown in Supplementary Table 3). All *L. ruminis* strains were grown in de Man-Rogosa-Sharpe (MRS; BD Difco, Le Pont-de-Claix, France) medium under anaerobic conditions using an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 24 hr. *B. acidifaciens* and *Bacteroides intestinalis* strains were grown in modified Gifu anaerobic medium (mGAM, Nissui Pharmaceutical, Tokyo, Japan) broth under anaerobic conditions using an AnaeroPack system at 37°C.

Growth assay of L. ruminis *supplemented with wakame or alginate as the sole carbon source*

B. acidifaciens was grown in 5 mL of mGAM (Nissui Pharmaceutical, Tokyo, Japan) broth for 24 hr. Gifu anaerobic medium (GAM) Semisolid without Dextrose (Nissui Pharmaceutical) was supplemented with 0.5% (w/v) wakame powder or Sodium Alginate 300-400 (Fujifilm Wako Pure Chemical, Osaka, Japan). After autoclaving at 105°C for 10 min, 500 µL of bacterial suspension was added to 5 mL of the medium and incubated under anaerobic conditions for 10 hr. The broth was then centrifuged at 8,000 × g for 10 min at 4°C, and the supernatant was collected, sterilized by 0.22 µm filtration, and designated as "wakame-fermented broth" or "alginate-fermented broth".

L. ruminis strains were grown in 5 mL mGAM broth for 24 hr. Then, 50 µL of the bacterial suspensions was added to 3 mL of wakame-fermented broth or alginate-fermented broth and incubated under anaerobic conditions. After 24 hr of cultivation, bacterial growth (OD_{660}) was monitored using a Mini Photo 518R photometer (TAITEC, Saitama, Japan).

Anti-inflammatory effect of L. ruminis *strains in porcine intestinal epithelial cells*

A single colony of each *L. ruminis* strain was transferred to 5 mL of MRS broth and cultured at 37°C for 24 hr under anaerobic conditions. For preculturing, 5 mL of MRS broth was inoculated with a 2% volume of bacterial suspension and incubated at 37°C for 24 hr. Then, a 2% volume of the culture of each strain was inoculated into 20 mL of fresh MRS broth and incubated at 37°C for 14 hr. Subsequently, the bacterial cells were centrifuged at 3,000 × g for 5 min, and the pellet was washed with 5 mL of phosphate-buffered saline (PBS, pH 7.4). The bacterial suspensions were centrifuged again (3,000 × g for 5 min), and the pellet was resuspended in 5 mL of PBS. Subsequently, bacterial cells were heat-treated for 90 min at 70°C. The number of bacterial cells was adjusted to 2.5 × 10^9 cells/mL under a microscope using a Petroff-Hausser counting chamber. The cells were stored at −30°C until use.

PIE #2 cells were seeded at 3 × 10^4 cells/well in 24-well type I collagen-coated plates (Sumitomo Bakelite Co., Tokyo, Japan)

and cultured for 3 days. After changing the medium, heat-treated *L. ruminis* strains (2.5×10^9 cells/mL) were added, and 24 hr later, each well was washed vigorously with medium at least three times to eliminate all stimulants. Then, cells were stimulated with lipopolysaccharide (LPS) (100 ng/mL; from *Escherichia coli* O55:B5 prepared by 0.22 µm filtration, L6529, Sigma, St. Louis, MO, USA) for 6 hr. Total RNA was collected, and cDNA was synthesized following the manufacturer's instructions (Takara, Kusatsu, Shiga, Japan). The expression of mRNA (CCL4, CCL5, and CXCL10) was measured using two-step real-time qPCR (Supplementary Materials and Methods).

Statistical analysis

Data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). All statistical analyses are described in the figure legends and the *Materials and Methods* section. Error bars in figures represent the standard deviation (SD), and p-values are shown in the figures. Multiple comparison tests indicated statistically significant differences ($p<0.05$) between values different symbols in the figures.

RESULTS

Development of an ex vivo batch-culturing system for the pig microbiome

The gut microbiota comprises a heterogeneous population, the composition of which is influenced by both host genetics and environmental factors [18, 23]. The gut microbiota plays a crucial role in nutrient utilization, energy harvesting, and carbohydrate metabolism, particularly in the breakdown of nondigestible polysaccharides. A thorough understanding of the impact of dietary interventions on gut microbiota is essential for maintaining gut homeostasis. Previous studies have mainly relied on *in vivo* experiments, which may not provide generalizable results, given the wide variation in microbiota composition among individuals and farms. To address these challenges, we aimed to develop an optimized *ex vivo* mixed culture medium that supports the growth of a large proportion of species from a given pig microbiome sample and is amenable to throughput evaluation.

To identify a medium that could support the growth of a batch culture with a composition similar to that of the original pig microbiome, pig feces that had been freshly collected from three donors, frozen, and then stored was cultured in nine different media deficient in microbiota-accessible sugar (Supplementary Table 2). DNA was extracted from all the samples, the V3–V4 region of the bacterial 16S rRNA gene was amplified, and the amplicons were sequenced (Fig. 1A). OTUs were inferred from the sequencing results, and the taxonomic composition was determined for each sample (Fig. 1B). Beta diversity (β-diversity) was used to generate a principal coordinate analysis (PCoA) plot with Bray–Curtis dissimilarity (Fig. 1C). The microbial community structure was found to be markedly different in media Nos. 1–3, 5, and 7–9 compared with the fecal samples, based on the permutational multivariate analysis of variance (PERMANOVA). Among all the tested media, media Nos. 4 and 6 showed the closest distance from the donor feces according to the Bray-Curtis distances (Fig. 1D). To better understand the microbiome diversity, differences between various media and individual donors were assessed using different methodologies (observed OTU richness, Shannon, and Simpson indices; Fig. 1E–1G). Indeed, high levels of variation were observed in the taxonomic compositions and diversities of the different media. The alpha diversity (α-diversity) of the microbiome was markedly lower in seven media (Nos. 1–3, 5, and 7–9) than in the donor feces, as indicated by the Shannon index (Fig. 1F). At the family level, media Nos. 4 and 6 largely matched the composition of the donor feces (Supplementary Fig. 1A).

The metabolism of seaweeds and their polysaccharides via intestinal bacteria has been known to impact SCFA profiles as well as the microbiome composition [45]. To understand the SCFA profiles of the media, we analyzed the concentrations of SCFAs in the media using high-performance liquid chromatography (HPLC). Acetate was the primary SCFA in the donor feces. The other major SCFAs were propionate and butyrate; succinate and lactate were rarely detected (Fig. 1H; Supplementary Table 4). The ratio of the major SCFAs (acetate/propionate/butyrate) was approximately 60/26/13. After 30 hr of cultivation, high variation was observed in the SCFA levels among the individual media (Fig. 1H). The acetate/propionate/butyrate ratio in medium No. 4 was 60/23/17, consistent with that in the fecal samples. In contrast, propionate and butyrate were not detected in medium No. 6, and the composition of SCFAs was remarkably different from that of the donor feces and medium No. 4 (Fig. 1H). Quantitative PCR (qPCR) revealed that the total number of bacteria in medium No. 6 was considerably lower than that in medium No. 4 (Supplementary Fig. 1B). Based on these results, among the nine media commonly used for gut microbiome cultivation, we selected modified medium No. 4 [39], as it supported the growth of a bacterial community that was most similar in composition and diversity to that observed in pig donor feces.

Ex vivo ***and*** **in vivo** ***validation of microbiome composition changes in response to wakame consumption***

We determined whether the changes in the microbial community and SCFA profiles after wakame consumption could be replicated in an *ex vivo* model supplemented with wakame powder. A previous wakame powder feeding study reported marked changes in several microbiome parameters, including an increase in *Lactobacillus* abundance and a decrease in *Enterobacteriaceae* (*E. coli*/*Shigella*) abundance in wakame-fed piglets [27].

Herein, fecal samples collected from eight donors were added to an *ex vivo* model supplemented with 0.2%, 1%, or 2% (w/v) wakame powder and subjected to 30 hr of anaerobic culture (Fig. 2A, left; Supplementary Fig. 2A). Next, we performed 16S rRNA gene sequencing of the bacterial communities derived from *ex vivo* media samples. We found that the changes in PCoA with Bray–Curtis exhibited significant dissimilarity over the amount of wakame (Supplementary Fig. 2B, 2C). The effects of wakame on the number and proportion of bacteria were assessed in *ex vivo* samples using the number of observed OTUs and the Shannon and Simpson diversity indices (Supplementary Fig. 2D–2F). The mean α-diversity scores increased with the amount of wakame powder, and a marked increase was observed in the 2% (w/v) wakame-supplemented medium.

In the *in vivo* study, sixteen pigs housed on the same farm were divided into two groups: wakame-fed and control (Fig. 2A, right). The first group was fed 1% (w/w) wakame powder ad libitum for 14 weeks. Similar levels of average daily diet consumption were observed between the two groups (wakame-fed group, 61.4 g/

weight [kg]; control group, 59.2 g/weight [kg]). During the experimental period, the average daily body mass gains for the wakame-fed and control groups were 762.5 ± 191.2 g and 809.4 ± 94.7 g, respectively. Based on 16S rRNA gene sequencing of the bacterial communities, the relative abundances of microbial OTUs at the phylum and genus levels for individual samples revealed that the communities exhibited moderate stability across all *in vivo* samples (Fig. 2B, Supplementary Fig. 3A).

To further understand the wakame-dependent microbiome diversity, we used the Bray–Curtis dissimilarity-based approach to evaluate the variation in wakame contribution between *ex vivo* and *in vivo* groups (Fig. 2C). Wakame-cultured *ex vivo* microbiomes were well separated from their controls (without wakame; Fig. 2C), and PERMANOVA revealed that the β-diversity community structure was extensively different in both wakame-supplemented *ex vivo* media and *in vivo* feces compared with that in the control (Fig. 2C, Supplementary Fig. 3B). Furthermore, ANOSIM based on the Bray–Curtis distance showed that the *ex vivo* microbiome had an ANOSIM R of 0.693 (p=0.038), while the *in vivo* microbiome had an R of 0.812 (p=0.012). This analysis revealed that wakame supplementation explained a remarkable portion of the variation in the gut microbial community composition both *ex vivo* and *in vivo*.

Next, we analyzed the patterns of SCFA profiles in *ex vivo* and *in vivo* samples. The major SCFAs were acetate, propionate, and butyrate in the *ex vivo* media, whereas butyrate was not detected in feces (Fig. 2D, Supplementary Fig. 4A). Notably, the SCFA profiles of wakame-cultured *ex vivo* media showed elevated succinate and lactate levels, whereas acetate and propionate levels were decreased (Supplementary Fig. 4B). These changes occurred in a concentration-dependent manner. Wakame-dependent trajectories of SCFA levels were also observed in wakame-fed feces (Supplementary Fig. 4A). Indeed, Bray–Curtis dissimilarity-based PCoA of the SCFA composition revealed wakame-dependent structural similarities (Fig. 2E). The composition of the wakame-cultured *ex vivo* media closely matched that of the wakame-fed feces (Supplementary Fig. 3C).

To further examine the relationship between SCFAs and microbial community structures in *ex vivo* and *in vivo* samples, we evaluated the association between microbiota clusters and SCFA types (Supplementary Table 5). The correlation coefficients between the percentage of bacteria present at the genus level and the production of each SCFA were calculated for the wakame-supplemented *ex vivo* media and *in vivo* feces. The results revealed that the top five bacterial genera that were positively correlated with individual SCFA levels were partially shared in common and included genera such as *Megasphaera* and *Olsenella* (acetate), *Anaerobranca* and *Mariniphaga* (propionate), *Phascolarctobacterium* (succinate), and *Lactobacillus* (lactate; Supplementary Table 5). Taken together, the microbiome diversity and SCFA composition suggest that the responses to wakame feeding due to metabolism by the gut microbiome can be captured by our established *ex vivo* model.

Characterization of Lactobacillus *species that were increased in the wakame-associated microbiome*

Based on the 16S rRNA gene sequencing data, we identified the top five bacterial genera that exhibited markedly different prevalences between the wakame-supplemented and control groups (Fig. 3A). The results revealed that the relative abundance of *Lactobacillus* was considerably increased in both wakame-supplemented *ex vivo* media and *in vivo* feces compared with that in the control (Fig. 3B). This increase was not observed in other *ex vivo* media (Supplementary Fig. 5). Notably, *L ruminis* was found to be extensively abundant among the detected *Lactobacillus* species in the wakame-associated *ex vivo* and *in vivo* microbiomes (Fig. 3C).

To determine the causal effect of the increased relative abundance of *L. ruminis* in the presence of wakame, five isolated *L. ruminis* strains from pig feces were cultivated in GAM with wakame powder as the sole sugar source. After 24 hr of anaerobic cultivation, the optical density (OD_{660}) values of the five strains of *L. ruminis* hardly changed in the wakame-supplemented medium compared with those in the basal medium (Fig. 3D). Alginic acid is the major polysaccharide in wakame, accounting for approximately 22% of its total weight (Supplementary Table 1). *Bacteroides* species degrade alginate to produce alginate oligosaccharides, which are utilized by *Faecalibacterium prausnitzii* as a sugar source via cross-feeding to promote growth [46]. We selected the *B. acidifaciens* JCM 10556^T strain harboring the alginate lyase homolog (WP_175630478.1). *B. acidifaciens* was identified by 16S rRNA gene sequencing of wakame-fed pig feces. *L. ruminis* strains were cultivated for 24 hr in cell-free supernatants of wakame- or alginate-supplemented GAM broth pre-fermented with *B. acidifaciens* (Fig. 3D, Supplementary Fig. 6). The results showed that pig fecal isolates 642, 450, and 425 exhibited remarkable growth in *B. acidifaciens* pre-fermented in both media, suggesting that the increase in *L. ruminis* abundance in the presence of wakame may be due to cross-feeding with *Bacteroides*.

Previous studies have reported that wakame supplementation can induce elevated levels of *Lactobacillus*, which, in turn, can regulate inflammation via the TLR4 signaling pathway [28]. *L. ruminis* also regulates chemokine production in intestinal epithelial cells [47, 48]. We evaluated the anti-inflammatory effects of *L. ruminis* on porcine intestinal epithelial cells (PIE #2; Fig. 3E–3G). After pre-culturing the PIE #2 cells, the five heat-treated *L. ruminis* strains were co-cultured for 24 hr. Subsequently, LPS, an inflammatory inducer, was added to the wells. After 6 hr, the mRNA expression level of each chemokine was determined using qPCR. Stimulation with LPS induced the mRNA expression of representative chemokines produced by the intestinal epithelium, namely CCL4, CCL5, and CXCL10 (Fig. 3E–3G). The addition of *L. ruminis* strains resulted in the strain-specific downregulation of LPS-induced chemokine mRNA expression. The type strain, *L. ruminis* JCM 1152^T (isolated from the bovine rumen), had a minimal impact on CCL4 and CCL5 expression and extensively decreased the expression of CXCL10. Interestingly, pig fecal isolates (16-7, 642, 450, and 425) exhibited distinct anti-inflammatory profiles across different strains. Notably, the 16-7 strain markedly reduced the expression of all tested chemokines compared with stimulation with LPS alone (Fig. 3E–3G). The results suggest that *L. ruminis* isolates increased in the presence of wakame and exerted an anti-inflammatory effect by modulating chemokine levels in the host.

DISCUSSION

In this study, we established *ex vivo* microbial communities derived from pig feces to assess the effect of feeding pigs a

A

Ex vivo

Bacteria	Relative abundance (%)		*p*-value
	Control	+wakame	
Lactobacillus	4.999	7.323	0.0088
Butyrivibrio	0.175	0.229	0.0151
Acetobacteroides	0.135	0.182	0.0410
Campylobacter	0.222	0.275	0.0419
Pseudobutyrivibrio	0.270	0.324	0.0638
Escherichia/Shigella	8.786	5.919	0.0058
Olsenella	1.203	0.930	0.0331
Anaerotruncus	0.105	0.090	0.0983
Megasphaera	6.316	5.327	0.1833
Gemmiger	0.114	0.137	0.2132

In vivo

Bacteria	Relative abundance (%)		*p*-value
	Control	+wakame	
Lactobacillus	4.866	7.509	1.32×10^{-6}
Clostridium_sensu_stricto	1.482	3.445	6.21×10^{-6}
Alloprevotella	1.141	2.242	1.08×10^{-5}
Treponema	1.802	2.441	2.58×10^{-5}
Methanosphaera	1.711	2.682	1.01×10^{-4}
Frischella	7.472	2.29	6.90×10^{-6}
Megasphaera	5.570	3.814	1.12×10^{-5}
Lachnospira	4.751	0.572	1.18×10^{-5}
Dialister	2.332	0.292	4.17×10^{-5}
Selenomonas	1.811	1.192	8.91×10^{-5}

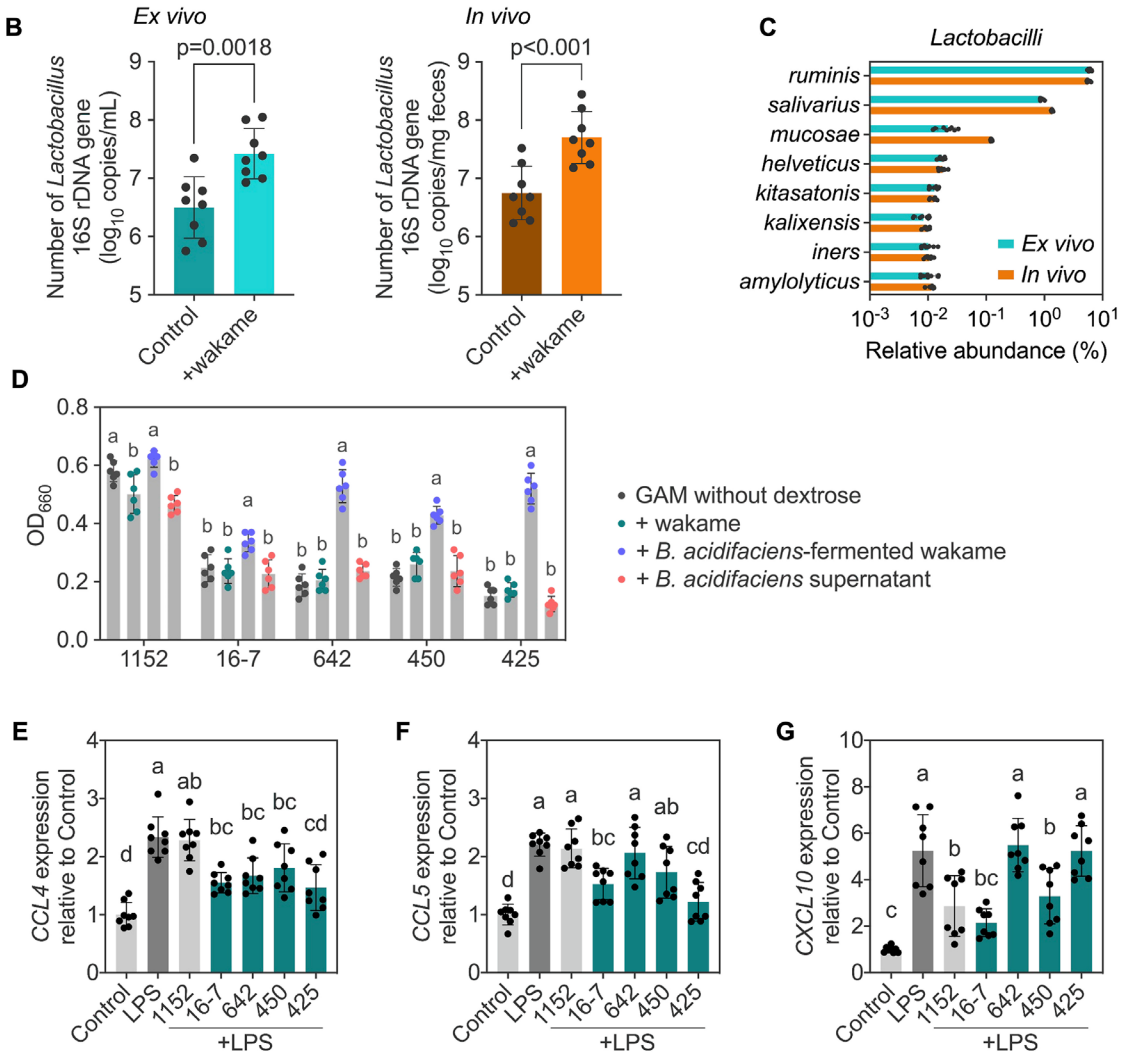

Fig. 3. Characterization of *Lactobacillus* species that were increased in the wakame-associated *ex vivo* and *in vivo* microbiome.

(A) The top five bacterial genera with significantly different prevalences between the wakame-supplemented and control groups based on 16S rRNA gene sequencing data. The significance of differences in relative abundance was determined based on the results of a Student's t-test. The red area indicates the bacterial species that increased in response to wakame supplementation, while the blue area represents the bacterial species that showed increases in the control group. (B) Relative abundances of the *Lactobacillus* in the *ex vivo* and *in vivo* samples, as detected by quantitative polymerase chain reaction (qPCR). Variations between groups were analyzed using Student's t-test (n=8 biologically independent cultures or feces). (C) *Lactobacillus* species-level distribution in the wakame-associated *ex vivo* and *in vivo* microbiome, based on *16S rRNA* gene sequencing data. (D) Growth capabilities of *L. ruminis* strains in the medium supplemented with wakame, *B. acidifaciens*-fermented wakame, or *B. acidifaciens* supernatant. The data show the optical density (OD_{660}) after culture for 24 hr. Different letters indicate significant differences as assessed by two-way ANOVA and Tukey's comparisons ($p<0.05$). The bars represent the average of two independent experiments with different batches of bacterial culture (biological replicates, n=3 per batch). (E–G) Expression of chemokines in porcine intestinal epithelial (PIE #2) cells treated with *L. ruminis* strains and challenged with lipopolysaccharide (LPS). Untreated PIE #2 cells were used as controls. The bars represent the average of two independent experiments with different wells (biological replicates, n=4 per test). Different letters indicate significant differences as assessed by Tukey's test ($p<0.05$).

GAM: Gifu anaerobic medium; ANOVA: analysis of variance.

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food by-product (specifically wakame) using a combination of microbial cultivation and *in vivo* experiments. Our approach differs from previous studies in several key aspects. First, we used an *ex vivo* culture model that did not require specialized culture devices. Unlike most previous studies, which focused on maintaining the bacterial community for extended periods (>48 hr) using a continuous culture model [39–41], our *ex vivo* culture model effectively captured the changes in the microbiome and SCFAs resulting from wakame supplementation while sustaining the microbiome for up to 25 hr. This was accomplished by screening the culture medium using a batch culture model. Importantly, our *ex vivo* model allowed us to explore a broader range of combinations, enabling us to expand the use of diverse donor feces and by-products.

Second, we investigated variations in the microbiome and SCFAs using an *ex vivo* model, with a specific focus on the effects of wakame feeding in pigs. Although several studies have attempted to mimic the composition of the donor fecal microbiome, our main objective was to use an *ex vivo* culture system to replicate the microbiome changes that occur when pigs are fed specific diets. This approach provides an opportunity to assess the suitability of food by-products as livestock feed. The introduction of wakame feed to pigs resulted in remarkable alterations in microbiome composition and concurrent fluctuations in SCFA concentrations. Although the *ex vivo* model did not completely emulate the bacterial composition observed *in vivo*, it effectively captured the trajectory of the microbiome shifts. Importantly, we successfully reproduced the changes in the SCFA composition both *in vivo* and *ex vivo*, with these alterations being dependent on the presence of wakame. This suggests that the *ex vivo* microbiome maintained the proportional ratios of SCFAs produced through wakame fermentation, while preserving functional redundancy.

Our previous studies have confirmed the occurrence of microbiome alterations following the supplementation of feed with wakame [11, 27, 28]. Additionally, investigations of pigs treated with wakame have shown an increased abundance of *Lactobacillus* spp. These *in vivo* findings regarding microbiome changes were further validated using an *ex vivo* model, suggesting the involvement of certain bacteria that efficiently metabolize wakame components through cross-feeding interactions. Notably, wakame supplementation led to a remarkable enrichment of *Lactobacillus* species, particularly *L. ruminis* isolates, and it was demonstrated that they had the ability to suppress chemokine expression levels in small intestinal cells. These results suggest that wakame holds promise as an effective livestock feed capable of modifying immune function by altering the pig microbiome. However, it is significant to note that microbiome data were obtained using a fecal culture model designed to mimic a pig anaerobic lumen environment, which is similar to that of the large intestine. Therefore, careful consideration is needed, particularly in terms of assuming distinct gastrointestinal environments for the small and large intestines.

Despite these significant advances, it is important to acknowledge the inherent limitations of the proposed approach. Our results do not directly assess the effects of various SCFAs and wakame components on the host. Further *in vivo* studies are necessary to evaluate the relevant biological parameters and establish the generality of this system. Moreover, it is essential to conduct validation experiments using donor feces from diverse backgrounds to account for variations in the microbiome resulting from environmental factors related to rearing conditions [23]. Therefore, the utilization of by-products in livestock feeds should be evaluated not only in terms of their impact on the microbiome but also for their effects on host homeostasis. Nonetheless, we believe that our *ex vivo* model serves as a valuable initial screening tool for validating by-products and assessing candidate feeds that have not been used previously, thereby providing insights into the intestinal environment of by-products as eco-feeds. The *ex vivo* gut microbiota model for pigs performed using a batch culture in this study was named "TUSIMM" (Tohoku University Swine Intestinal Microbiota Model). We believe that assessment of the livestock microbiome using TUSIMM is invaluable for promoting the active utilization of by-products as feed, thereby contributing to the development of an environmentally sustainable livestock industry.

DATA AVAILABILITY

The authors confirm that data supporting the findings in this study are available within the article and the indicated supplementary materials. The gut microbiome data is openly available at doi: 10.17632/bjyvny8g4x.1.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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