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研究課題名（和文）Development of novel Immunosynbiotics with wakame utilizing immunobiotics and immunogenics

研究課題名（英文）Development of novel Immunosynbiotics with wakame utilizing immunobiotics and immunogenics

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研究成果の概要（和文）：抗生物質の過剰使用による耐性問題が深刻であり、効果的な代替手段が求められています。イムノシンバイオティクス（免疫調節微生物と免疫調節食品材料の組み合わせ）は有望な代替手段です。ワカメは免疫調節食品とされ、ワカメを食べた豚の腸内から分離された乳酸菌は免疫調節乳酸菌と考えられます。ワカメを飼料に加えることで豚の免疫反応が改善され、細菌叢が変化することがわかりました。本研究では、乳酸菌はゲノム解析で異なるワカメ利用能力が示されました。また、選抜された乳酸菌は胃液での耐性が示されたインビボ研究では、この株を使用したワカメ発酵飼料を与えた豚で有益なラクトバチルス菌の増加と病原菌の減少が確認されました。

研究成果の学術的意義や社会的意義
本研究は、イムノシンバイオティクスが抗生物質代替として有効であることを実証し、ワカメと乳酸菌の組み合わせが豚の腸内環境を改善することを明らかにしました。特に、免疫反応の向上と腸内微生物叢の変化で、持続可能な畜産方法への貢献が期待されます。ゲノム解析による乳酸菌のワカメ利用も微生物の理解を深める成果です。ワカメを飼料にすることで抗生物質使用を減らし、耐性菌の拡大を防ぐことが可能です。また、東日本大震災後の宮城県経済復興に寄与し、地域経済の活性化にもつながります。持続可能な畜産業の発展に向けた新しい飼料開発として、広く社会に良い影響を与えることが期待されます。

研究成果の概要（英文）：In order to minimize the impact of antibiotic resistance in livestock production, microbial materials with immuno-modulatory functions have been proposed to be promising candidates for antibiotic substitutes. Immunosynbiotics, a combination of immunomodulatory microorganisms (immunobiotics) and immunomodulatory food materials (immunoprebiotics), may have synergetic effects to protect animals from pathogenic bacteria and viruses. Wakame is a prospective material for immunoprebiotics, promoting growth of lactobacilli, which have been proved to have in vitro immunomodulatory ability. To improve the effectiveness of immunosynbiotics in the gut health of pigs, I aim to determine sugar utilization profiles and host immune modulatory patterns using biochemical, genomic, and immunological approach. Finally, the efficacy of the developed immunosynbiotics will be evaluated using in vitro and in vivo porcine models to improve the healthy growth of antibiotic-independent porcine production.

研究分野：Animal Science

キーワード：Immunobiotics　Wakame　L. salivarius　immunogenic

１．研究開始当初の背景

The gastrointestinal tract infections caused by pathogens have been considered as major health problems in weaning piglets and have caused severe economic loss on farms and food safety problems. Antibiotics have been widely applied in intensive livestock production. In 2012, 70% of antibiotics were consumed by animals and it was estimated that by 2030, global consumption of antibiotics in livestock production was predicted to increase by two-thirds (1). However, a growing number of antibiotics including colistin and virginiamycin have been banned or restricted worldwide due to the severe problem of antibiotic resistance. Thus, researchers across the globe are urged to search for effective alternatives. Here, we proposed that “immunosynbiotics”, a combination of immunobiotics and immunoprebiotics with synergistic effects when used in feed, would be one of the most promising candidates for substitutes of antibiotics to solve the severe problem of antibiotic resistance in the world.

Immunobiotics are identified as those probiotic bacteria that promote health through the beneficial modulation of the mucosal immune system. We recently reported that immunobiotics *Ligilactobacillus salivarius* could modulate the innate immune response related to bacteria surface structure. These *L. salivarius* were isolated from the intestine of wakame-fed pigs. When pigs were fed with wakame, the ratio of *L. salivarius* of gut microbiota has been significantly increased (2). Thus, the porcine intestinal *L. salivarius* is considered an interesting source of potential immunobiotic bacteria for the application in the pig industry.

The seaweed wakame (*Undaria pinnatifida*) has been considered as immunoprebiotic since it contains biologically active components such as fucoidan, alanine acid that could modulate the immune system function, including anti-inflammatory and antiviral activities (3). On the other hand, Miyagi prefecture ranked the 2nd place in wakame production in Japan. However, along with the human consumption of the edible parts, side products of wakame waste have been dramatically increased. As cash crop, production of wakame could be one of the best alternatives of economic recovery in Miyagi prefecture after the 2011 Tohoku earthquake. Wakame is rich in cellulose and hemicellulose as major polysaccharides and only a few lactic acid bacteria can metabolize these polysaccharides. Recently, in order to improve the healthy growth and production of livestock, wakame have been studied as feed supplements in livestock, especially in pigs. The addition of wakame to feed proved to increase the percentage of peripheral blood natural killer (NK) cells and CD3+CD4-CD8+ lymphocytes and alter microbiota in the porcine large intestine (4). Wakame could be a promising candidate for immunoprebiotics and with the combination of immunobiotics, the development of immunosynbiotics could be carried out.

２．研究の目的

Since the above background and research development, the proposed work is aimed to ❶ select and characterize potentially beneficial immunosynbiotic lactobacilli strains isolated from the intestine of wakame-fed pigs by evaluating their abilities of utilizing digested wakame saccharides and the tolerance to gastric juice under the wakame presence. These functional studies will be complemented with ❷ comparative genomic evaluation using the complete genome sequences of these lactobacilli candidates that demonstrated wakame utilization abilities as immunosynbiotic properties. Immunogenics related to immunomodulatory abilities will be determined by knocking out the specific genes relating to either immunomodulatory ability or wakame utilization ability. The ultimate objective of the proposed work is ❸ in vivo study of evaluating the improvement of intestinal disorder symptoms of piglets by feeding with immunobiotic fermenting wakame and thereby contribute to the antibiotic-independent healthy growth in livestock production and healthy life.

３．研究の方法

1. Whole genome sequencing and comparative genomic analysis: Whole genome of selected *Lactobacillus* spp. will be analyzed and comparative genomic analysis will be performed to identify unique genes possibly related to hydrolysis or immuno-modulatory ability will be identified.
2. Knockout/genome modification of related unique genes: The Lactobacillus genome will be edited to create the strain deficient of putative genes (knockout mutant) using the CRISPR/Cas9 genome editing system according to Wang et al. (ACS Synth. Biol.).

3. *In vitro* immune assay and wakame utilization research: Immunomodulatory ability of both the mutant and knockout/genome-modified strains will be evaluated by in vitro immune assay by using both porcine bronchial and intestinal epithelial cell lines. The OD value and viable bacterial count will be evaluated utilization ability in mutant and knockout/genome modified Lactobacillus strains in previously developed wakame broth.
4. *In vivo* study of the application of immunosynbiotic to porcine: The knockout/genome-modified Lactobacillus fermented wakame will be administered orally with feeds to piglets. Clinical improvement of the symptoms of the intestinal disorder will be investigated. Intestine, fecal and other organ samples will also be collected to investigate the change in gut microbiota and immunoregulator changes.

４．研究成果

(1) Eight lactobacilli strains selected in previous studies were able to grow better in wakame-based broths developed in this study (Fig. 1) containing enzyme-treated wakame leaf or wakame stalk with the addition of B vitamins. The comparative genomic analysis revealed that the differences between the strains were related to their different abilities to utilize enzyme-treated wakame(Fig. 2).

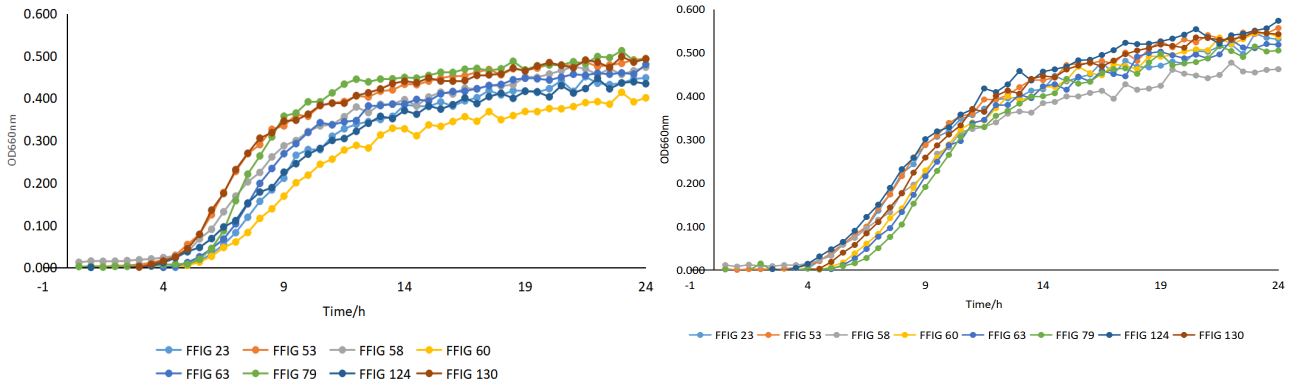

Figure 1. Optical density (OD) of *Ligilactobacillus salivarius* isolated from the intestinal mucosa of wakame-fed pigs fermented in leaf wakame (A) and stalk wakame (B). The results represent data from three independent experiments.

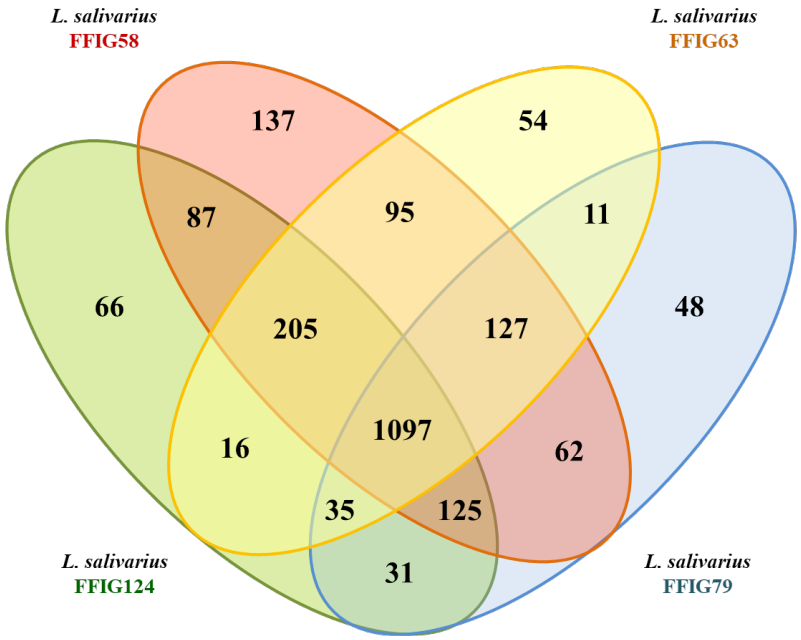

Figure 2. Genomic comparison of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. Four “wakame assimilative phenotypes” were defined according to the ability of *L. salivarius* FFIG strains to efficiency of assimilating the saccharide in developed wakame broth.

(2) One strain that has been proven to have adhesion ability to porcine intestinal epithelial cells (Fig. 3) could survive under the simulated gastric juice in the presence of wakame because

the fiber contained in wakame could protect the cells from the severe environment of acid (Fig. 4).

(3) The growth and storage conditions have various effects on the characteristics of lactic acid bacteria, especially high-temperature and low-temperature stress, and differences in carbon sources not only affect the division and proliferation of lactic acid bacteria but also change their surface characteristics, affecting their adhesion to host cells and immune-regulating properties. The incubation condition of the selected strain was determined (Fig. 5). In future research, the strain under determined incubation conditions will be provided for the *in vivo* study.

(4) We conducted an in vivo study by feeding 36 piglets with wakame fermentation using this promising strain. Furthermore, our observations demonstrated that the abundance of beneficial Lactobacillus bacteria increased, while pathogenic strains such as *E. coli* decreased (data not yet summarized).

The results indicate that the potential applications of immunosynbiotics in promoting overall well-being are exciting areas for further investigation and could lead to practical ways to enhance health in the future.

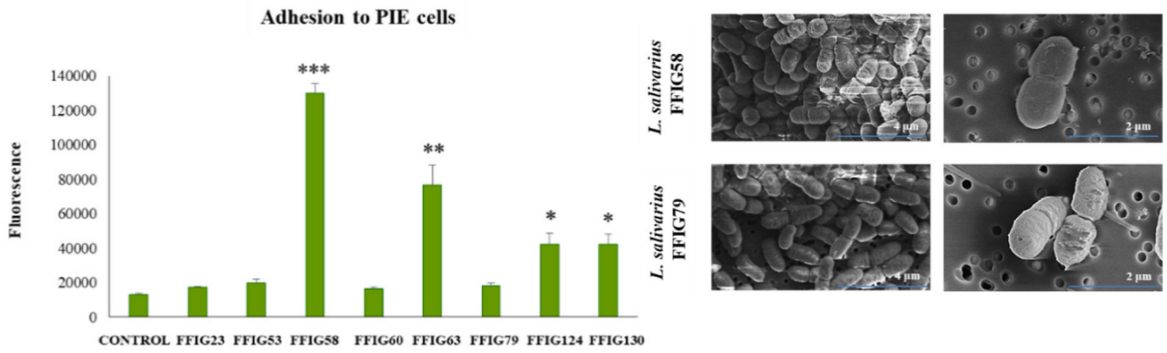

Figure 3. Adhesion of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs to porcine intestinal epithelial (PIE) cells. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the control PIE cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Scanning electron microscope (SEM) analysis of *L. salivarius* FFIG58 and *L. salivarius* FFIG79.

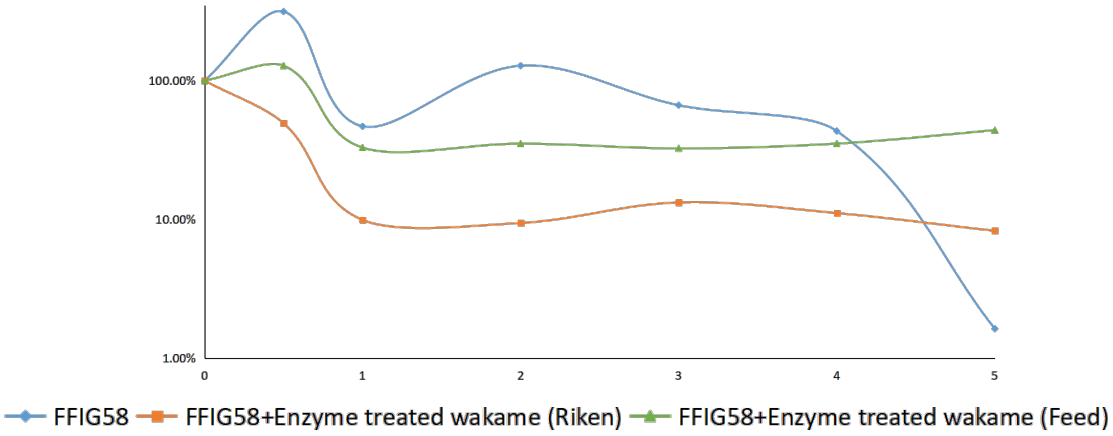

Figure 4. Viable bacteria index of *Ligilactobacillus salivarius* FFIG58 when FFIG58 were stimulated in simulated gastric juice. Samples were taken at 0, 0.5, 1, 2, 3, 4, 5 h. Viable bacteria index was measured compared to viable bacteria counted at 0 h.

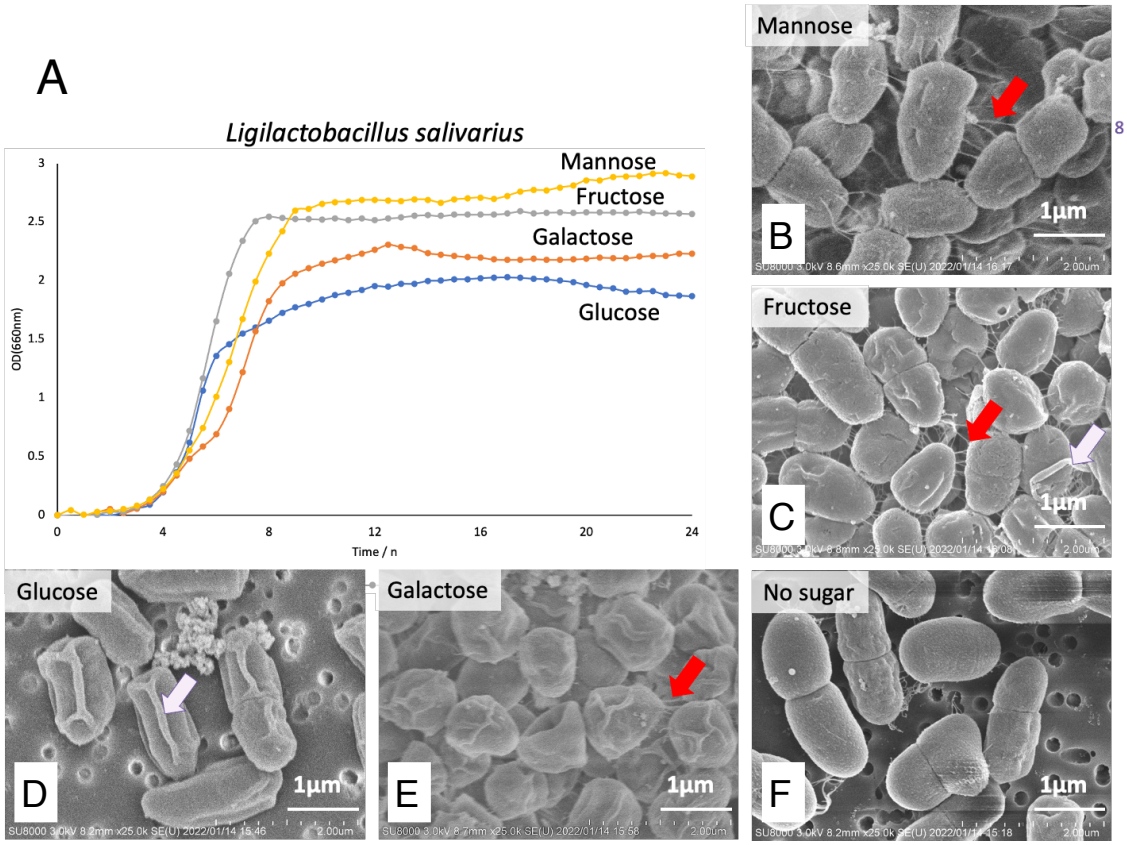

Figure 5. Optical density (OD) of *Ligilactobacillus salivarius* isolated from the intestinal mucosa of wakame-fed pigs fermented in MRS broth with different carbon sources(A). Scanning electronic microscope photo of *Ligilactobacillus salivarius* in MRS broth with different carbon sources. (B) Mannose (C) Fructose (D) Glucose (E) Galactose (F) No sugar. Scale bar: 1μm.

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3. Liu, F. *et al.*, Fucoidan extract derived from Undaria pinnatifida inhibits angiogenesis by human umbilical vein endothelial cells. *Phytomedicine*, 2012.
4. Shimazu T. *et al.*, Addition of Wakame seaweed (*Undaria pinnatifida*) stalk to animal feed enhances immune response and improves intestinal microflora in pigs. *Anim. Sci. J.*, 2019.

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　1件）

１．著者名 Ohgi Ryusuke、Saha Sudeb、Zhou Binghui、Sakuma Taiga、Sakurai Mitsuki、Nakano Yuhka、Namai Fu、Ikeda-Ohtsubo Wakako、Suda Yoshihito、Nishiyama Keita、Villena Julio、Kitazawa Haruki	４．巻 15
２．論文標題 In vitro evaluation of the immunomodulatory and wakame assimilation properties of Lactiplantibacillus plantarum strains from swine milk	５．発行年 2024年
３．雑誌名 Frontiers in Microbiology	６．最初と最後の頁 1-10
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fmicb.2024.1324999	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計3件（うち招待講演　0件／うち国際学会　1件）

１．発表者名 Binghui Zhou, AKM Humayun Kober, Muhammad Shahid Riaz Rajoka, Wakako Ikeda-Ohtsubo, Julio Villena, Haruki Kitazawa
２．発表標題 Survival of immunobiotics in the GI tract for immunosynbiotic development
３．学会等名 酪農科学シンポジウム2022
４．発表年 2022年

１．発表者名 周 冰卉, 劉 瑛琦, 櫻井 美月, AKM　Humayun Kober, Muhammad Shahid Riaz Rajoka, 大坪 和香子, Julio Villena, 北澤 春樹
２．発表標題 Basic research on growth improvement of immunosynbiotic
３．学会等名 日本畜産学会第130回大会
４．発表年 2022年

１．発表者名 Binghui Zhou, Ryusuke Ohgi, Taiga Sakuma, Sudeb Saha, Mitsuki Sakurai, Yuka Nakano, Fu Namai, AKM, Humayun Kober, Wakako Ikeda-Ohtsubo, Keita Nishiyama, Haruki Kitazawa
２．発表標題 Isolation and immunosynbiotic characterization of lactic acid bacteria from swine milk to generate a Lactobacillus library
３．学会等名 FEMS2023（国際学会）
４．発表年 2023年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関